

MATRIX METALLOPROTEINASES AS POSSIBLE BIOMARKERS  
OF OBSTRUCTIVE SLEEP APNEA SEVERITY

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By

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## Abstract

Upper airway collapse in obstructive sleep apnea (OSA) is associated with intermittent hypoxia, which resembles ischemia-reperfusion (IR). Oxidative stress in IR has been shown to increase matrix metalloproteinases (MMPs) action and lead to adverse cardiovascular consequences in animal models. The results of previous studies on circulating MMP level in OSA are inconsistent. Oxidative stress in OSA might also directly contribute to kidney injury. The alteration in urinary MMP levels have been previously shown in acute and chronic renal injury, but there is no data about MMP levels in urine of OSA patients.

The aim of this study is to determine if serum and urine MMPs in OSA patients are associated with OSA severity and CVD in OSA.

The study is a part of a multi-center Canadian trial performed through the Canadian Sleep and Circadian Network. OSA subjects (n=124) were recruited from the Sleep Disorders Center (Saskatoon City Hospital, Saskatchewan, Canada) after in-lab polysomnography (PSG). Controls (n=26) were subjects referred to the Center who did not have OSA. Severity of OSA was categorized based on apnea/hypopnea index (AHI) according to American Academy of Sleep Medicine criteria. Level of hypoxemia was expressed as oxygen desaturation index (ODI). MMP-2 and MMP-9 activities were measured in blood and urine samples with gelatin zymography method. Albumin-to-creatinine ratio (ACR - indicator of glomerular damage) was calculated from urinary albumin and creatinine. Mann-Whitney U and Kruskal-Wallis tests were used for statistical analysis. Multivariable linear regression was used to control for potential confounders.

Serum MMP-2 activity was associated with OSA severity/level of hypoxemia in OSA, even after adjustment for age, sex, body mass index (BMI), and CVD ( $p = 0.004$ ). In stratified analysis, the association between serum MMP-2 and AHI remained significant only for patients with CVD ( $p = 0.009$ ). Serum MMP-9 was associated with white blood cell count, after adjustment for AHI, age, sex, BMI and CVD ( $p < 0.001$ ). Urinary MMPs did not show any association with OSA severity. Urinary MMP-2-to-creatinine in OSA was associated with ACR, independently of hypertension and diabetes ( $p = 0.022$ ). There were no differences in MMP activities between OSA patients with CVD and without CVD.

The results suggest that in future studies serum MMP-2 might be considered as biomarker of OSA severity, whereas MMP-9 may be a marker of inflammation in OSA. Urinary MMP-2 was associated with glomerular damage in OSA. Prospective studies in larger sample size are needed to confirm and delineate observed associations.

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## List of abbreviations

AASM – American Academy of Sleep Medicine  
AHI – apnea/hypopnea index  
BMI – body mass index  
BNP – brain natriuretic peptide  
CIH – chronic intermittent hypoxia  
CPAP – continuous positive airway pressure  
Cr – creatinine  
CRP – C-reactive protein  
CVD – cardiovascular disease  
ECM – extracellular matrix  
EEG – electroencephalogram  
EKG – electrocardiogram  
EMG – electromyogram  
EOG – electrooculogram  
ELISA – enzyme-linked immunosorbent assay  
IL-6 – interleukin 6  
IRI – ischemia/reperfusion injury  
MMP – matrix metalloproteinase  
NF- $\kappa$ B – nuclear factor kappa B  
ODI – oxygen desaturation index  
OSA – obstructive sleep apnea  
PSG – polysomnography  
RDI – respiratory disturbance index  
ROS – reactive oxygen species  
RNS – reactive nitrogen species  
siRNA – small interfering RNA (ribonucleic acid)  
SNP – single nucleotide polymorphism  
SpO<sub>2</sub> – blood oxygen saturation  
SpO<sub>2</sub> <90% - time of blood oxygen saturation below 90%  
TIMP – tissue inhibitor of matrix metalloproteinases  
VIF – variance inflation factor  
WBC – white blood cells

# 1 INTRODUCTION

Obstructive sleep apnea (OSA) is the most common sleep-related breathing disorder, characterized by recurrent episodes of upper airway obstruction leading to intermittent hypoxia and arousals [1]. Nearly 1 billion individuals aged 30–69 years worldwide are estimated to have OSA, with prevalence exceeding 50% in some countries [2]. The prevalence of OSA has been increasing [3]. In the biennial Canadian Health Measures Survey (2016-2017) 6.4% of adult Canadians reported they had been diagnosed with sleep apnea [4], whereas the prevalence among adults reported in the Canadian Community Health Survey in 2009 was 3% [5].

Due to poor sleep quality, patients with OSA frequently experience daytime somnolence and fatigue, which is associated with worse work performance and higher risk of motor vehicle crashes [1,6]. Moreover, severity of OSA is associated with an increased risk of cardiovascular disease (CVD) [7]. Current evidence suggests that it is predominantly the hypoxic burden that contributes to the emergence of CV complications of OSA which affect primarily those with severe disease. The main challenge of clinical management of OSA is to identify which patients are at higher risk of these adverse consequences. Finding a simple non-invasive biomarker for assessing OSA severity and hypoxic burden would allow to stratify risk and to implement proper therapy early in the course of disease to prevent development and/or progression of the adverse cardiovascular complications [8].

The pathophysiology of OSA comorbidities is likely multifactorial. Chronic intermittent hypoxia (CIH) in OSA results in inflammation, endothelial dysfunction, elevated oxidative stress, and sympathetic nervous system activation [9]. Considering the complexity of OSA, many biomarkers, including markers of inflammation, oxidative stress, catecholamines and adhesion molecules, have been already proposed, however, the results are still inconclusive [8].

Recurrent episodes of apneas/hypopneas in OSA cause a desaturation-reoxygenation sequence. It resembles ischemia/reperfusion injury (IRI) which is associated with excessive production of reactive oxygen species (ROS)/reactive nitrogen species (RNS) [10]. ROS/RNS are highly reactive molecules that, if at high quantities, can cause damage to the nucleic acids, lipids and proteins, and in turn contribute to numerous pathological conditions, including CVD. The imbalance between ROS and antioxidant defence is called an oxidative stress [11].

Oxidative stress during ischemia/reperfusion or asphyxia causes an increase in matrix metalloproteinases (MMPs) action and leads to adverse cardiovascular consequences [12–14]. An upregulation of oxidative stress markers and impaired antioxidant capacity in OSA has been

previously shown [10]. MMPs action can therefore be a molecular mechanism linking OSA with oxidative stress and CVD.

There is some evidence of associations between MMPs and OSA severity, which was described in a recent systematic review (see Chapter 2) [15]. However, the published studies are limited by their experimental design, small subject numbers, and/or lack of adjustment for potential confounders. The vast majority of published results in humans investigated MMP-9 levels. However in animal models there is strong evidence of role of MMP-2 in oxidative stress [16–18].

Although OSA is associated with a loss of kidney function [19] and microalbuminuria [20], so far there is no data on MMPs in the urine of OSA patients. Increased risk of loss of kidney function in OSA can be related to OSA co-morbidities (such as hypertension, diabetes), but oxidative stress often seen in OSA patients might directly contribute to kidney injury. Thus, some biochemical changes (including protein release and modification in protein profile) in urine of OSA patients may be observed.

## **1.1 Obstructive sleep apnea**

### **1.1.1 Pathogenesis and pathophysiologic consequences**

OSA is a disorder characterized by episodes of markedly reduced (hypopnea) or absent (apnea) airflow during sleep, caused by the decrease of upper airway dilating muscle activity superimposed on a narrow upper airway [1]. These episodes are usually accompanied by blood oxygen desaturation, fluctuations in blood pressure and heart rate, and are typically terminated by micro-arousals [21,22].

OSA risk factors include male sex, age 40-70 years, obesity, large neck, craniofacial abnormalities, smoking, use of sedatives before sleep, and select endocrinological disorders associated with abnormal upper airway anatomy or physiology (hypothyroidism, acromegaly) [6]. Several published studies have established the role of genetic factors in the development of OSA [23]. The pathophysiological factors for OSA that appear to be influenced by genetic factors include differences in body fat distribution, control of breathing, and craniofacial and upper airway morphology [22,23].

OSA significantly impacts quality of life. Among major sequelae are habitual snoring, excessive daytime sleepiness due to poor sleep quality, daytime fatigue, poor work performance, and higher risk of motor vehicle crashes [6]. Furthermore, OSA has numerous other serious health consequences. Patients with OSA are at higher risk of developing

cardiometabolic diseases, including systemic and pulmonary hypertension, heart failure, atherosclerosis, coronary artery disease, stroke, diabetes and metabolic syndrome [24].

Moreover, intermittent hypoxia, activation of sympathetic nervous system and OSA comorbidities (hypertension, diabetes) may lead to development of chronic kidney disease and/or its progression in OSA [25]. OSA has been previously shown to be associated with kidney injury [20] and accelerated loss of kidney function [19].

#### 1.1.2 Diagnosis and classification of severity.

The gold standard for OSA diagnosis is in-lab polysomnography (PSG). It is a comprehensive overnight study during which neurologic and cardio-respiratory parameters are recorded, including air flow, respiratory effort, blood oxygen saturation, electrocardiogram (EKG), electrical activity of the brain (EEG), eyes (EOG), muscle tone (EMG) and body movements [6].

According to current American Academy of Sleep Medicine (AASM) criteria [26], severity of OSA is categorized based on apnea/hypopnea index (AHI) which is the number of apneas and hypopneas per hour. Apnea is defined as a cessation of breathing for a minimum of 10 seconds. Hypopnea is defined as a decrease of airflow for more than 10 seconds associated with oxygen desaturation of 3% or an arousal. Severity of OSA is defined as follows:  $\geq 5/h$  (mild OSA),  $\geq 15/h$  (moderate OSA),  $\geq 30/h$  (severe OSA) [26].

#### 1.1.3 Treatment

OSA treatment depends on the severity of disease. Patients with OSA can largely benefit from reduction in risk factors, namely weight loss, avoidance sedatives before sleep, or sleeping on the side. The therapy of choice for patients with moderate-to-severe sleep apnea is continuous positive airway pressure (CPAP) therapy. CPAP is a device that provides a positive airway pressure to upper airway during sleep to ensure the airway stays open. CPAP therapy reduces daytime sleepiness and other symptoms, and improves sleep and quality of life [27]. Moreover, it has been shown that CPAP reduces the risk of fatal and non-fatal cardiovascular events in OSA patients [28].

## 1.2 Matrix metalloproteinases

MMPs are a family of over 20 calcium-activated and zinc-dependent endopeptidases [29]. They are responsible for degradation of extracellular matrix (ECM), but it has been shown that MMPs act also at the intracellular level [30].

MMPs were discovered in 1962, when Gross and Lapiere tried to establish how the metamorphosing frog tadpole lost its tail. They found a collagenolytic activity in amphibian tissue, and it was later named matrix metalloproteinase-1. MMPs are found in most living organisms [31].

Based on substrate specificity and structural homology MMPs are classified into the following groups: collagenases, gelatinases, stromelysins, matrilysins, membrane-type (MT)-MMPs and other non-classified MMPs [32].

### 1.2.1 Gelatinases (MMP-2 and MMP-9)

#### 1.2.1.1 *Substrate specificity and function*

Gelatinases are the most studied MMPs. MMP-2 is a constitutive enzyme found in almost all cell types, including endothelium, vascular smooth muscle cells, cardiomyocytes and connective tissue cells [16,33]. MMP-9 is expressed in endothelium, cardiomyocytes, smooth muscle cells, but mainly in leukocytes, and it is easily induced by pro-inflammatory cytokines [16,34].

Gelatinases are known to cleave native collagen type IV, V, VII and X, XI, as well as elastin and fibronectin [29]. The first evidence of intracellular localization and action of MMP-2 was shown in 1997 [35]. The group discovered an MMP-2-mediated pathway of human platelet aggregation, and described the ultrastructural localization and cellular translocation of MMP-2 in the stimulated human platelets [35]. Following this study, several new intracellular actions of MMPs were revealed, including degradation of big endothelin [36], monocyte chemoattractant protein-3 [37] and cardiac contractile proteins including troponin I [38], titin [39] and myosin light chain type 1 and 2 [14,40].

Gelatinases play an important role in a variety of physiological processes including morphogenesis, cartilage and bone repair, wound healing, cell migration and angiogenesis [16]. The dysregulation of MMPs' action leads to development of many pathological conditions, such as chronic ulcers, rheumatoid arthritis, cancers and CVD [41]. They have been shown to play a role in development of dilated cardiomyopathy, cardiac remodeling after myocardial infarction and atherosclerotic plaque rupture [42]. MMPs, apart from the involvement in

chronic (days-weeks) processes [43], have also been found to increase their activity within minutes, for example in acute IRI in the heart [44].

#### *1.2.1.2 Structure*

MMP-2 and MMP-9 structure includes a signal peptide, amino-terminal propeptide, a zinc-binding domain containing the catalytic site, and a carboxyl-terminal hemopexin-like domain [32]. Additionally, MMP-2 and MMP-9 have fibronectin-like gelatin-binding domain which contributes to their substrate specificity [34]. MMP-2 and MMP-9 were assigned to MMP family due to their sequence homology with MMP-1: a highly conserved sequence PRCGXPD in the propeptide, and the sequence HEXGHXXGXXH in the catalytic domain [30].

The signal peptide is responsible for guiding the transport of enzymes into the endoplasmic reticulum. The propeptide domain maintains the proenzyme in an inactive state (by interaction between the cysteine residue in a propeptide with the active zinc ion site in the catalytic domain). Catalytic domain, apart from one catalytic zinc, contains one structural zinc, and three calcium ions. The catalytic zinc ion is stabilized within the catalytic domain by three histidine residues (located in the highly conserved sequence HEXGHXXGXXH). [30,34].

The hemopexin-like domain facilitates the interaction between MMPs and tissue inhibitor of metalloproteinases (TIMPs), and it is not required for catalytic activity [30,34].

#### *1.2.1.3 Regulation of activity*

MMPs are expressed as latent enzymes. They are activated by the disruption of bond between a cysteine thiol residue and the active zinc ion site. This can be achieved by two mechanisms. First, several proteases (such as plasminogen, kallikrein, tissue plasminogen activator, trypsin as well as MMPs themselves) can cause proteolysis of the propeptide domain. Secondly, ROS can directly change a protein conformation exposing catalytic site of MMP [45].

During activation by proteolytic cleavage of proenzyme, the active form is then shorter by approximately 10 kDa compared to proenzyme. In case of activation by oxidants without proteolytic cleavage, activated form is same size as the latent form.

MMP activity may be regulated at multiple levels including transcription, secretion, activation or suppression by specific and nonspecific inhibitors [45].



Due to the fact that MMP-2 is constitutively expressed by many cells, its activity depends more on post-translational events than transcriptional regulation. Unlike MMP-2, MMP-9 gene is highly inducible by pro-inflammatory cytokines and oncogene products, which may increase its transcription by as much as 100-fold [34].

MMPs are naturally inhibited by  $\alpha$ 2-macroglobulin and the endogenous tissue inhibitors of metalloproteinases (TIMPs). Human  $\alpha$ 2-macroglobulin is a plasma glycoprotein with the function of binding of MMPs, and then their removal from the circulation via scavenger receptors on the phagocytes [30,46].

TIMPs are a family of small proteins (20-30 kDa), which inhibit MMPs by forming non-covalent complex in a 1:1 molecular ratio. To date, four TIMP isoforms have been identified (TIMP-1, TIMP-2, TIMP-3, TIMP-4). They are broad-spectrum inhibitors of MMPs, but they show some differences in specificity, for example TIMP-1 strongly binds to MMP-9, whereas TIMP-2 preferentially modulates activity of MMP-2 [34,47].

Synthetic inhibitors of MMPs, such as o-phenanthroline, hydroxamates and tetracyclines, inhibits MMPs by chelating zinc ion in their catalytic site. Currently, doxycycline is the only clinically approved inhibitor of MMPs [45].

## 2 OBJECTIVES AND HYPOTHESIS

My overall aim of this project was to investigate the relationship between OSA and MMPs. Based on the literature presented, it was hypothesized that urinary and serum MMPs may be biomarkers of hypoxemia severity and the severity of OSA. This study was a step towards identification of a biomarker for risk stratification, triage of OSA patients, and a marker which can provide the opportunity for more targeted therapy for OSA.

*Objective 1:* To determine if adult OSA (obstructive sleep apnea) patients have a distinct serum and urine MMPs (matrix metalloproteinases) levels when compared to controls.

*Hypothesis 1:* Serum and urine levels of MMP-2 and MMP-9 are increased in OSA patients

*Objective 2:* To determine if serum and urine MMP-2 and MMP-9 levels differ in regards to the severity of OSA.

*Hypothesis 2:* Serum and urine levels of MMP-2 and MMP-9 correlate with the severity of OSA.

*Objective 3:* To verify if adult OSA patients with cardiovascular comorbidities have a distinct serum and urine MMPs level when compared to OSA patients without cardiovascular comorbidities.

*Hypothesis 3:* Adult OSA patients with cardiovascular comorbidities have an increased serum and urinary MMPs level when compared to OSA patients without cardiovascular comorbidities.

### **Format of the thesis**

I will begin this thesis with a systematic review of articles on MMPs in OSA (Chapter 3). A systematic review was prepared in order to critically appraise the existing studies on the topic, and it is included in the thesis to provide a thorough background on the topic and demonstrate the gaps in knowledge that exist in this area. This systematic review represents the work I completed preparing for the research around the three objectives as part of my MSc program. The review has been published in 2019 in the journal *Sleep Medicine Reviews* [15]. Following this, I describe the methods used to address the three objectives. Finally, I discuss the results of the thesis in the context of the existing literature and draw conclusions.

In order to help explain potential associations observed around urinary MMP activities, kidney function and level of kidney injury was assessed. However, it can be considered a secondary outcome as it was not part of the original objectives.

### 3 LITERATURE REVIEW

Franczak A, Bil-Lula I, Sawicki G, Fenton M, Ayas N, Skomro R. Matrix metalloproteinases as possible biomarkers of obstructive sleep apnea severity - A systematic review. *Sleep Med Rev* 2019;46:9–16. DOI: 10.1016/j.smr.2019.03.010.

#### 3.1 Summary

Obstructive sleep apnea is an underdiagnosed sleep-related breathing disorder affecting millions of people. Recurrent episodes of apnea/hypopnea result in intermittent hypoxia leading to oxidative stress. Obstructive sleep apnea is considered an independent risk factor for cardiovascular disease but the exact pathophysiology of adverse cardiovascular outcomes of obstructive sleep apnea has not been fully elucidated. Matrix metalloproteinases (MMPs) have been associated with both oxidative stress and cardiovascular diseases. Hypoxic conditions were shown to influence MMP expression, secretion and activity. Moreover, matrix metalloproteinases contribute to ischemia/reperfusion injury. Therefore, action of matrix metalloproteinases can provide a possible molecular mechanism linking obstructive sleep apnea with oxidative stress and cardiovascular disease. The aim of this paper was to review the current evidence of association between matrix metalloproteinases and obstructive sleep apnea with focus on hypoxemia and severity of obstructive sleep apnea.

Key words: obstructive sleep apnea, sleep disordered breathing, matrix metalloproteinase, oxidative stress, cardiovascular disease, systematic review

#### Glossary of terms

AHI – apnea-hypopnea index

BMI – body mass index

CIH – chronic intermittent hypoxia

CRP – C-reactive protein

CVD – cardiovascular disease

ELISA – enzyme-linked immunosorbent assay

IL-6 – interleukin 6

MMPs – matrix metalloproteinases

NF- $\kappa$ B – nuclear factor kappa B

ODI – oxygen desaturation index

RDI – respiratory disturbance index

ROS – reactive oxygen species

siRNA – small interfering RNA (ribonucleic acid)

SNP – single nucleotide polymorphism

SpO<sub>2</sub> – blood oxygen saturation

SpO<sub>2</sub> <90% - time of blood oxygen saturation below 90%

## **3.2 Introduction**

### **3.2.1 Obstructive sleep apnea – clinical characteristics, prevalence and cardiovascular complications**

Obstructive sleep apnea (OSA) is the most common sleep-related breathing disorder. It is characterized by recurrent episodes of airflow cessation (apnea) or reduction (hypopnea), caused by the decrease of upper airway dilating muscle activity during sleep superimposed on a narrow upper airway. These abnormalities impair normal ventilation during sleep and result in hypoxemia and sleep fragmentation as a consequence of frequent respiratory-related arousals leading to poor sleep quality, daytime somnolence and adverse effects on quality of life [1]. Cognitive complaints, fatigue and mood disorders are frequently observed. Furthermore, OSA is associated with worse work performance and higher risk of motor vehicle crashes [6].

OSA has been considered a major public health problem [48]. It affects all age groups but increases in prevalence with age. The prevalence of OSA is estimated as 13% among adult males and 5.6% among adult females [3]. OSA rates have been increasing worldwide along with the prevalence of obesity [49].

Despite the high prevalence and increased social awareness of OSA, this disorder still remains under-diagnosed. OSA is a condition with an insidious onset which can contribute to its delayed diagnosis. Data from a Canadian cross-sectional survey showed that only 5.1% of those at higher risk for OSA reported being referred to a sleep laboratory [50]. The delay in diagnosis and treatment leads to development of numerous complications. It has been shown that patients with untreated OSA are at higher risk of neurocognitive impairment, Alzheimer's disease and a wide range of cardiovascular conditions [9,51] such as systemic and pulmonary hypertension, heart failure, atherosclerosis, coronary artery disease and stroke [52,53]. The mechanisms which have been suggested to contribute to these adverse outcomes include sympathetic nervous system activation, inflammation, endothelial dysfunction and oxidative stress [9].

### 3.2.2 OSA as model of chronic intermittent hypoxia

The narrowing or collapse of upper airway is frequently associated with a repetitive transient decrease in arterial oxygen saturation [54]. Therefore, one of the hallmarks of OSA is CIH (chronic intermittent hypoxia). The hypoxic events and arousals can lead to sympathetic nervous system activation, which contributes to peripheral vasoconstriction and triggers an increase in arterial blood pressure and heart rate. Systemic hypertension can in turn promote cardiac hypertrophy, diastolic dysfunction and potentially lead to congestive heart failure. Furthermore, increased negative intra-thoracic pressure, caused by the airway occlusion and resultant increased respiratory effort, generates an additional mechanical stress (increases in both pre-load and after-load) on the heart resulting in progression of cardiac remodeling. In addition to vasoconstriction, hypoxia leads to oxidative stress, inflammation and endothelial dysfunction, all of which are important element of atherosclerosis development. The adverse effect of CIH on the cardiovascular system may be a reason of premature death of OSA patients, mainly by a greater risk of myocardial infarction [55].

The exact pathophysiology of the adverse consequences of OSA is not fully-understood and is likely multi-factorial. However, it has been hypothesised that matrix metalloproteinases (MMPs) may be an important marker of chronic intermittent hypoxia.

### 3.2.3 Matrix metalloproteinases as markers of intermittent hypoxia

MMPs are proteolytic enzymes, which take part in remodeling of extracellular matrix and have also been shown to have a biological action within the cell as well [38]. They are essential for the wide range of physiological processes; their dysregulation may contribute to development of pathological conditions including CVD (cardiovascular disease) [16]. They have been shown to play a role in development of dilated cardiomyopathy, cardiac remodeling after myocardial infarction and atherosclerotic plaque rupture [42]. Recent evidence suggests that MMPs contribute not only to long-term remodeling processes but also to acute ischemia-reperfusion injury in the heart [44].

MMPs are synthesized as inactive zymogens by inflammatory cells, fibroblasts and endothelium. They are activated by the disruption of bond between a cysteine thiol residue and the active zinc ion site. This can be achieved by two mechanisms. First, several proteases (such as plasminogen, kallikrein, tissue plasminogen activator, trypsin as well as MMPs themselves) can cause proteolysis of the propeptide domain. Secondly, reactive oxygen species (ROS) can directly change a protein conformation exposing catalytic site of MMP [45]. Oxidative stress

during ischemia/reperfusion or asphyxia causes an increase in MMPs action and leads to adverse cardiovascular consequences [12–14]. Recurrent episodes of apneas/hypopneas in OSA cause a desaturation-reoxygenation sequence that resembles ischemia/reperfusion injury which is associated with excessive production of reactive oxygen species (ROS) and upregulation of oxidative stress markers and impaired antioxidant capacity in OSA [10]. In addition, the results of studies on human and animal cell cultures have shown that MMPs expression, secretion and activity are induced by hypoxic conditions [56,57], which resemble those present in OSA patients, thus suggesting a potential role of MMPs' in OSA.

ROS, generated during CIH, can directly activate MMPs. Moreover, the MMPs activity can also be affected at the transcriptional level. CIH-mediated NF- $\kappa$ B (nuclear factor kappa B) pathway activation is an example of such regulation [58,59]. NF- $\kappa$ B upregulates not only numerous inflammatory cytokines and adhesion molecules, but also plays a role in the regulation of MMPs transcription [17,60]. Bond et al. have also shown that NF- $\kappa$ B is necessary to upregulate MMPs secretion by vascular smooth muscle cells [60]. The activation of NF- $\kappa$ B along with MMPs action can be a molecular mechanism linking OSA with oxidative stress, inflammation and in turn cardiovascular complications. The potential modes of action of MMPs in response to chronic intermittent hypoxia are summarized in Figure 3.1.

Moreover, some studies report different single nucleotide polymorphisms (SNP) in the promoter region of MMPs in OSA patients [61–63], which may influence MMPs expression. However, the results of these studies are inconsistent. It was shown that MMP-9 SNP (1562C/T) was associated with higher risk of OSA in Chinese [62], but not in Turkish population [63]. Moreover, another study in Turkish population showed that allele frequencies of MMP-9 1626C/T SNP significantly differ between OSA patients with CVD compared to those without CVD. However, MMP-9 genotype was not associated with MMP-9 level in serum [61]. MMP-2 SNP (-1306C/T) has not been shown to be associated with risk of OSA [62].

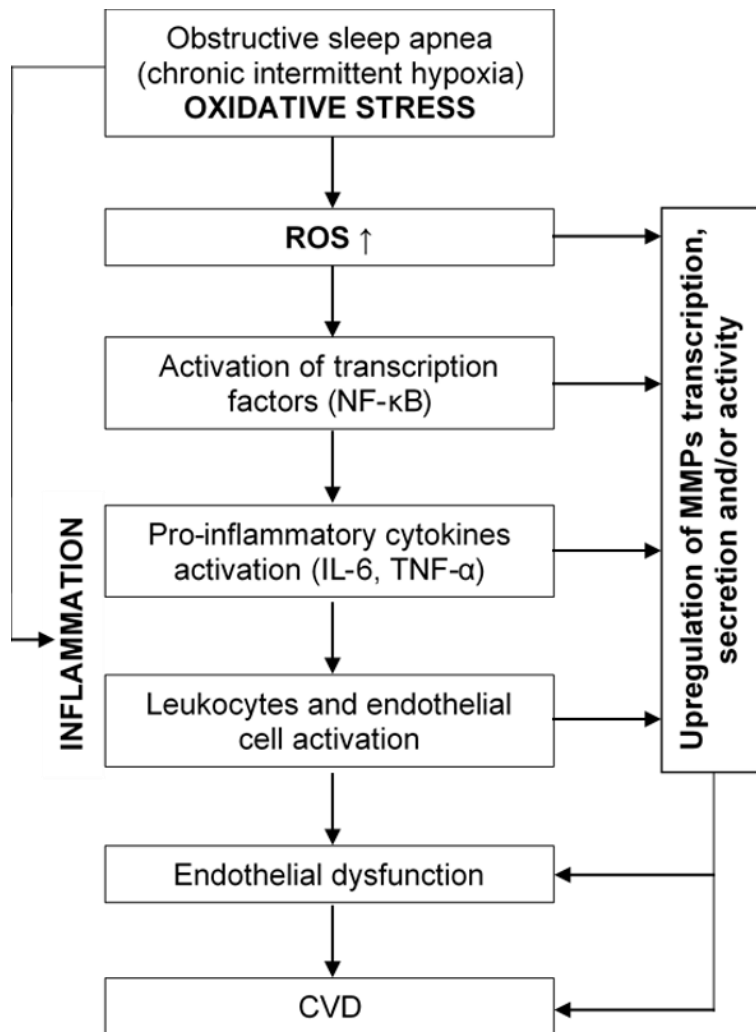


Figure 3.1 Potential MMP (matrix metalloproteinases) modes of action in response to chronic intermittent hypoxia.

CVD, cardiovascular disease; IL-6, interleukin 6; ROS, reactive oxygen species; TNF- $\alpha$ , tumor necrosis factor  $\alpha$ .



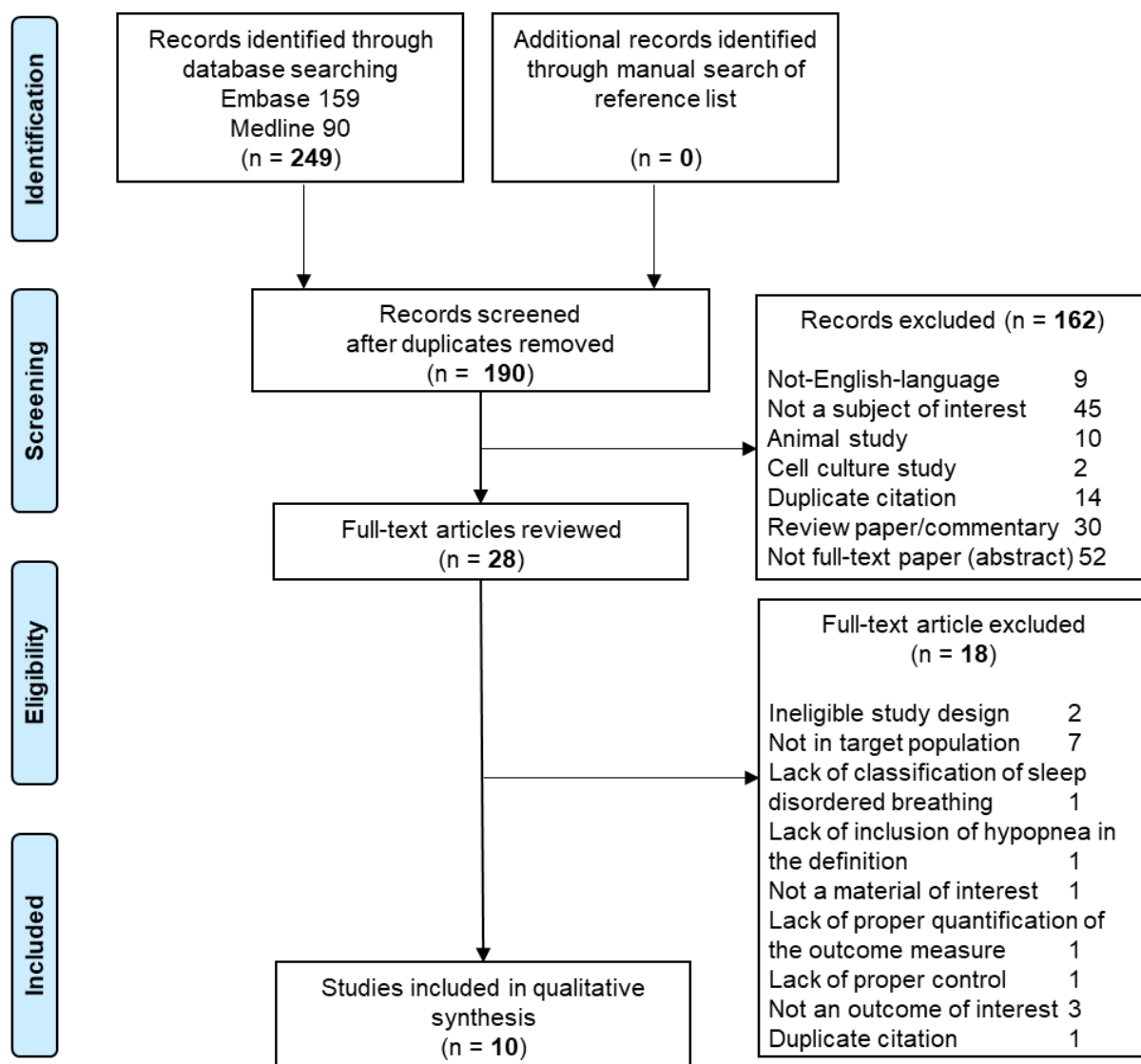
### **3.3 The aim of the review**

OSA can be considered a model of chronic intermittent hypoxia (CIH). Therefore, based on several lines of aforementioned evidence that MMPs play a role in hypoxia and CVD, it could be hypothesized that MMPs can be biomarkers of severity of hypoxemia, severity of OSA and may potentially be useful biomarkers of cardiovascular complications in OSA.

Considering the complexity of cardiovascular comorbidities in OSA, finding biomarkers which allow detection of patients at higher risk is a great challenge for sleep apnea research and clinical management. Although a lot of effort has already been expended in searching for possible biomarkers, the results are still inconclusive. Blood-based markers of inflammation (interleukin 6 (IL-6), C-reactive protein (CRP), high sensitivity CRP and tumor necrosis factor- $\alpha$ ), hemodynamic cardiac stress (N-terminal-B-type natriuretic peptide), myocardial injury (cardiac troponins) as well as indicators of antioxidant capacity and oxidative stress (thioredoxin, superoxide dismutase, malondialdehyde) have been proposed [64–68]. In this paper, we have reviewed the available literature about MMPs in this regard.

### **3.4 Methods**

This systematic review followed the Preferred Reporting Items for Systematic Reviews and Meta-Analysis PRISMA Checklist [69]. Our PICO question was: In patients with diagnosed moderate to severe OSA (P), will the severity of OSA (I) be associated with elevated levels of MMPs in biological samples (O) in comparison with patients without OSA or those with mild OSA (C)? Only studies involving human OSA subjects (adult and children) published in English were included. We have reviewed EMBASE (05/12/2018) and Medline (via Ovid – including in-process and other non-indexed citations; 05/12/2018) – see search details in Appendix A. Duplicates were removed through EndNote, and then titles and abstracts were reviewed manually by two authors (AF, RS). After initial screening, 2 investigators (AF, RS) reviewed independently the full-text manuscripts. References from the retrieved manuscripts were verified manually by two authors (AF, RS) to see if we missed any articles suitable for inclusion in this review. The process for selecting studies is provided in the flow chart (see Figure 3.2). The study quality was assessed with the modified Newcastle-Ottawa Scale (see Appendix B).



From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(7): e1000097. doi:10.1371/journal.pmed1000097

Figure 3.2 Flow diagram.

### 3.5 Results

Our search results revealed overall 249 articles. After excluding studies which did not meet our criteria (see Figure 3.2) 10 manuscripts were reviewed (see Table 3.1). All of these concerned MMP-9 and MMP-2.

#### 3.5.1 MMP-9 in adult OSA patients

Our review identified 8 publications that addressed the question of MMP-9 levels and activity in adult OSA patients. In all those articles, MMP-9 was analyzed in blood (serum, plasma, monocytes isolated from peripheral blood). The vast majority of results (five out of eight) showed that an increased blood MMP-9 level was associated with OSA and its increase was more notable in patients with severe OSA [56,70–73].

Tamaki et al. have shown that the production of MMP-9 by peripheral blood monocytes of patients with both severe and mild/moderate OSA was significantly higher compared to control; however there were no difference between severe and mild/moderate OSA [71].

Nevertheless, Bonanno et al. did not report any significant association between serum MMP-9 level and OSA, however, in severe OSA, a trend to increase MMP-9 level was observed [74]. Maeder et al. [75] and Volná et al. [76] also did not report any significant differences in blood MMP-9 concentrations in regards to OSA severity (defined by AHI (apnea-hypopnea index)).

The results of the study carried out by Hopps et al. provided some evidence of association between MMPs and oxidative stress in OSA. In this study a positive correlation between plasma levels of MMP-9 and lipid as well as protein peroxidation markers was found [77]. ROS can activate MMPs and indeed, Tazaki et al. showed that serum MMP-9 activity was elevated in patients with OSA compared to controls. Moreover, that activity was positively correlated with AHI [72].

A positive correlation between MMP-9 and level of hypoxemia expressed as ODI (oxygen desaturation index) and/or  $SpO_2 < 90\%$  (time of blood oxygen saturation below 90%) was shown in five studies [56,70,72,73,76]. Moreover, a negative correlation between MMP-9 and mean oxygen saturation was found [70,76]. All these results are in accordance with findings that hypoxic condition can induce MMP-9 expression, secretion and activity [56] which indicate that oxidative stress, one of the crucial factors of CVD, may contribute to pathophysiology of OSA by stimulation of MMPs action.

Bearing in mind that MMP-9 can be upregulated by pro-inflammatory cytokines, a positive correlation between serum levels of MMP-9 and IL-6 (interleukin 6) and TNF- $\alpha$  found in OSA patients by Tazaki et al. is not surprising [72]. Also, Ye et al. found a positive correlation between serum MMP-9 concentration and CRP level [73]. Moreover, Wang et al. [56] suggested that MMP-9 may be a predictor of CVD in OSA patients. During up to five-year long follow-up of 35 patients without hypertension, 12 of them developed hypertension and three patients had left ventricular hypertrophy. Increased serum level of MMP-9, OSA severity (defined by AHI) and decreased lowest SpO<sub>2</sub> were risk factors for new hypertension onset. All patients who developed left ventricular hypertrophy had an elevated serum levels of MMP-9.

In summary, the preponderance of evidence suggests that circulating MMP-9 level is increased in patients with OSA and this increase is related to OSA severity [56,70–73], inflammation [72,73] and/or severity of hypoxemia [56,70,72,73,76].

### 3.5.2 MMP-9 in OSA among children

There is only one study concerning MMP-9 in children with OSA. Kaditis et al. [78] showed that plasma MMP-9 level was correlated with CRP and BMI (body mass index), but there was no association between MMP-9 and severity of OSA. The vast majority of children (92.5%) were less than 10 years old. The AHI ranged from <1 episode/hour in patients without OSA to approximately 3 episodes/hour in mild OSA and 13 episodes/hour in moderate-to-severe OSA patients. The ODI ranged from  $0.9 \pm 0.6$  to  $15.6 \pm 14.1$  episodes/hour, depending on the OSA severity. There were no significant differences between MMP-9 level in children with and without OSA, which is in contrast to most studies in adults. This finding could be explained by the fact that children with OSA often do not have as advanced hypoxemia as adult patients. Therefore, the molecular CIH-dependent mechanisms of MMPs stimulation in children OSA patients may not be activated. The authors concluded that the primary reason for elevated MMP-9 level in blood of children with OSA may be inflammation which results from factors other than hypoxemia.

### 3.5.3 MMP-2 in adult OSA patients

Current literature on the subject of MMP-2 is more limited and inconclusive. Our search revealed only four published papers concerning MMP-2 in adults with OSA and no studies in children. MMP-2 was analyzed in serum or plasma in three manuscripts [70,74,76] and one

article investigated the presence of MMP-2 in superior pharyngeal constrictor muscle specimens (however, MMP-2 protein was not detectable [79]). Only one study [70] reported an increased MMP-2 plasma level in OSA patients; however, without any correlation with OSA severity (defined by AHI) [70]. Another study showed no association between OSA and blood levels of MMP-2 [76]. Interestingly, Bonanno et al. reported that MMP-2 level was lower in patients with severe OSA (here defined as respiratory disturbance index (RDI) > 30) compared to subjects with RDI < 30 [74]. In summary, the evidence linking MMP-2 and OSA severity is scarce with limited data in adults and no studies in children with OSA.

### **3.6 Discussion**

OSA is the most common sleep-related breathing disorder, which still remains underdiagnosed [50]. It leads to numerous cardiovascular complications which affect quality and length of patients' life [10]. Given that, early diagnosis is crucial to implement proper therapy early in the course of the disease to prevent adverse consequences related to OSA severity - decreasing incidence of CVD events among others. However, solely clinical criteria are insufficient to identify patients at higher risk for cardiovascular events. This review suggests that MMP-9 may be promising biomarker in this regard.

The preponderance of evidence suggests an association between MMPs (particularly MMP-9) and severity of OSA. Five out of eight studies that examined relationship between MMP-9 and OSA showed that MMP-9 level was elevated in patients with OSA in comparison with patients without OSA or those with less severe OSA [56,70–73]. Additionally, another study [74] reported a trend to increase MMP-9 level in severe OSA, however, without statistical significance (this may have been due to the lack of statistical power). Moreover, data from the reviewed studies suggests that MMP-9 may be potentially an indicator of level of hypoxemia, as in a few studies MMP-9 level was found to positively correlate with ODI and SpO<sub>2</sub> <90% [70,72,73,76].

The literature on the subject of MMP-2 in OSA is limited to four papers, therefore drawing any conclusions about MMP-2 association with OSA is difficult. However, there is the strong evidence of association between MMP-2 and oxidative stress in animal studies [16–18] and thus future studies of MMP-2 in larger samples of OSA patients should be considered, given the fact that recurrent episodes of apnea/hypopnea result in intermittent hypoxia and in turn lead to oxidative stress [10].

### 3.6.1 Study limitations

Our search has provided important insight into association between MMP level and OSA severity, however, the results of current literature on this subject are still preliminary. In most of the studies, sample sizes were small ( $N = 50-70$ ), which may have affected statistical powers of the study and different indexes (AHI, RDI) were used to express OSA severity (see Table 3.1).

Moreover, OSA definition varied across the studies. Most studies defined OSA by an  $AHI \geq 5/\text{hr}$ , however, some used the cut-off of  $AHI \geq 10/\text{hr}$  or  $RDI \geq 5/\text{hr}$  (see Table 3.1). Some of the studies used 3% desaturation to score hypopnea [76,78], others 4% [71,73–75], while Hopps et al. used a 3% or 4% drop in oxygen saturation, depending on % in reduction of breathing [70]. Three of studies did not note the cut-off for oxygen desaturation at all [56,72,79].

Furthermore, only six of studies used the gold standard for OSA diagnosis (see Table 3.1), and very few controlled for confounders. MMPs have been shown to have an impaired pattern in various CVDs, inflammatory diseases and metabolic syndrome [42,80]. MMP levels may depend on age and gender [81]. However, in only four studies [71–73,78] the potential confounders (age, gender, BMI, CRP concentration [78]; BMI, age [71,73]; BMI, waist circumference, waist/hip ratio [72]) were adjusted for in the analysis. Although some authors tried to address presence of confounders in the study design by excluding patients who have had CVD or systemic infections, CVD were often not well-defined [71,72,74,78] or limited to heart failure [75]. Two studies did not address the issue of confounding at all [70,77]. Hopps et al. [70] not only did not control for it, but enrolled in the study group patients with OSA and CVD, whereas control subjects were free of medical illnesses which were however not defined in detail.

Furthermore, majority of study designs were either cross-sectional or case-control in their design. Only one study was a cohort in its design to determine if MMP-9 level can be a predictor for CVD development and demonstrated possible role of MMP in development of CV complications [56]. However, numerous limitations of this study should be listed. First, sample size was small ( $n = 47$ ), and cohort chosen for follow-up constituted only 35 subjects. Second, OSA diagnosis was based on cardiorespiratory study (which is not a gold standard). It is also not clear from the paper if patients enrolled for follow-up were free from CVD (if assuming so, it still remains unknown how CVD was defined).

Last but not least, there are also some methodological limitations of reviewed studies. Only one study [72] used two techniques (enzyme-linked immunosorbent assay (ELISA) and

gelatin zymography) for MMP-9 measurement, whereas others used only one (ELISA or another immunoassay technique). It should be emphasized that ELISA and zymography differ significantly in regard to the principle of methodology. More precisely, immunoassay technique quantifies protein concentration based on reaction of antigen and antibody, whereas gelatin zymography is based on the gelatinolytic (proteolytic) activity of the enzyme and the latter one is more important from biological point of view. Moreover, in methods based on interaction of antigen and antibody (ELISA) there are many limitations associated with specificity and selectivity of the antibodies used for the detection of antigen, whereas zymography allows for detection of specific enzyme based on its catalytic activity and molecular weight [82,83].

Despite a number of limitations, the association between MMPs and OSA seems to be likely, especially in the light of biological plausibility. An elevated MMP-9 level in OSA patients with more severe disease compared to those with less severe or without OSA is in accordance with suggested contribution of oxidative stress and inflammation to OSA development [54], because MMPs' expression, secretion and activity may be modulated by hypoxia and inflammation [17,56]. Keeping in mind these feasible links between OSA, oxidative stress, inflammation, CVD and MMPs (see Figure 3.1), MMPs seem to be promising proteins for identification OSA patients at higher risk of CVD. Apart from the above-mentioned cohort study of Wang et al. [56], additional report showed that serum MMP-9 level was significantly increased in OSA patients with CVD (coronary artery disease, hypertension and cardiac arrhythmia) compared to those without CVD [61].

The studies in animal models also provide the evidence on the CIH-modulated MMP response and its potential association with CVD. Some authors have shown that CIH induce cardiac fibrosis in rats. Fibrosis results from an imbalance in extracellular matrix turnover which can be explained by observed significant changes in MMP levels in hearts of rats subjected to CIH [17,84–86]. However, analogously to human studies, results regarding the direction of these changes are not consistent [17,84–86].

The observations from the animal studies cannot be directly applied to humans, considering the anatomical and biochemical differences between species and the limitations of animal models to mimic the human OSA pathophysiology. Only one model applied the airway obstruction which is an integral component of OSA [86]. Furthermore, MMPs were not analyzed in body fluids, but in cardiac tissue. Moreover, various time of CIH was applied which also can be an explanation of observed differences in direction of changes in MMP levels. Also, the effect of short-term hypoxia varies from the long-term effect [87] and in the above-

mentioned papers the time of protocol varied from 7 days to 5 weeks with different pattern of hypoxia/normoxia cycles.

However, the animal studies, similarly to the human ones reviewed in this paper, provide some evidence that CIH has an impact on MMP response. Due to MMP's substrate specificity with respect to extracellular matrix proteins, it can lead to cardiac fibrosis and in consequence, CVD. Cardiac remodeling is associated with cardiac dysfunction which may manifest itself as malignant ventricular arrhythmias, heart failure and myocardial infarction complications [42], all of which have an increased prevalence in OSA patients. In addition, patients with OSA commonly develop left ventricular hypertrophy [88].

Given MMPs possible contributions to OSA adverse cardiovascular consequences, MMPs may also become a therapeutic target. Wang et al. showed that doxycycline, administered to rats for four weeks while receiving CIH, attenuated atrial fibrosis induced by CIH by modulation of MMP-2 and MMP-9 expression [85]. In the studies on ischemia/reperfusion injury in rat hearts it was shown that doxycycline prevented MMP-2-induced troponin I degradation and improved cardiac mechanical function [38,44]. Doxycycline, analogically to other studied MMP inhibitors such as o-phenanthroline and hydroxamates, inhibits MMPs non-selectively by chelating zinc ion in catalytic site of MMPs. Currently, doxycycline is the only clinically approved inhibitor of MMPs [45]. However, in the randomized controlled trial in patients undergoing coronary artery bypass graft surgery with cardiopulmonary bypass, doxycycline (20 mg) did not improve myocardial stunning following the surgery, despite the fact that the atrial MMP-2 activity was decreased. Authors suggested that higher dose of doxycycline should be considered in the next larger trials [89]. Lin et al. showed that MMP-2 siRNA (small interfering RNA), by specific inhibition of MMP-2 expression and activity, led to protection of contractile function of isolated rat cardiomyocytes subjected to chemical ischemia [90]. Due to increasing interest in the molecular therapy and precision medicine, there is a growing potential for the use of MMP-2 siRNA in clinical trials [91].

In conclusion, there are plausible biological mechanisms linking an increase in MMP levels and OSA and the preponderance of evidence from this systematic review suggests that MMPs (especially MMP-9) is elevated in OSA patients. These studies however are limited by their study design, small subject number and lack of adjustment for confounders. Although there are a few negative studies, MMPs seem to be promising predictors of hypoxemia and severity of OSA. It is possible therefore that they may be useful markers of adverse cardiovascular outcomes in OSA which appear to be mediated by hypoxemia. However, the



results of studies published on this subject are inconclusive and, thus, further research is needed to delineate the exact role of MMPs in OSA, particularly in the subset of patients with CV complications.

Future studies should be performed in larger samples of both children and adult populations, should be cross-sectional and prospective in their design, should adequately control for confounders and must be adequately powered to detect a difference in MMP levels.

## Practice Points

Matrix metalloproteinases are important molecules in obstructive sleep apnea research, because:

- Animal studies suggest involvement of MMP in models of CIH and their role in cardiovascular disease.
- The majority of studies suggest increased MMP levels (MMP-9 in particular) in patients with obstructive sleep apnea.
- The existing studies suggest presence of association between blood level of MMP-9 and obstructive sleep apnea severity in adults.
- Increased MMP levels in OSA patients may result from higher level of hypoxemia and therefore, MMPs could be a molecular mechanism linking OSA with oxidative stress and cardiovascular disease.
- There is very limited literature on this subject in children.
- There are no prospective studies evaluating the role of MMPs in predicting cardiovascular complications in OSA patients.
- Existing studies suffer from methodological weaknesses, especially in their approach to handling confounding.

## Research Agenda

- Future research should be aimed at delineating the role of matrix metalloproteinases in obstructive sleep apnea with focus on their associations with hypoxemia, severity and cardiovascular outcomes of the disease.
- Larger sample sizes are required in future studies.
- The current gold standard (i.e. in-lab PSG) should be used for diagnosis of OSA.
- Severity of the disease should be categorized according to American Academy of Sleep Medicine criteria.
- Future studies should be long term and must be powered to detect difference in MMP levels.
- More studies of MMP in children with OSA are needed.

### **3.7 Acknowledgements**

The authors wish to acknowledge grant support from Canadian Sleep and Circadian Network. The Canadian Sleep and Circadian Network (CSCN) is national in scope and is committed to scientific excellence in the generation of new knowledge and its translation. The CSCN was developed through a grant from the Canadian Institutes in Health Research combined with funds from partners and stakeholders. The network is inclusive, multidisciplinary and multi-thematic. It strives to facilitate effective interactions and collaborations between the scientific community, patients and stakeholders. It also has a training and career development mandate for the national sleep and circadian research community. It promotes the sharing of ideas, tools, methods and resources, and the dissemination of research outcomes. The CSCN's vision is to mobilize the healthcare community to adopt an integrated approach towards improving outcomes and treatment of patients with sleep disorders, with a first focus on Obstructive Sleep Apnea.

#### **Conflicts of interest**

The authors do not have any conflicts of interest to disclose.

Table 3.1 Overview of studies of MMPs in OSA.

Author, reference number	Study design	Number of subjects	Type of diagnostic study *	Type of MMPs	Material	OSA definition	Compared groups	The direction of changes in MMP level**		Comment
								MMP-2	MMP-9	
Bonanno et al. [74]	Cross- sectional	50	Type 1	MMP-2 MMP-9	Serum	RDI $\geq 5$	Severe (RDI $>30$ ) vs non-severe	↓	↑ #	
Dantas et al. [79]	Case-control	51	Type 1	MMP-1 MMP-2	Superior pharyngeal constrictor muscle specimens	AHI $\geq 5$	Cases vs controls	MMP-2 was not detectable (IHC).		Variable MMP-1 expression (IHC) within the same muscle fiber and between patients.
Hopps et al. [70]	Case-control	79	Type 3	MMP-2 MMP-9	Plasma	AHI $\geq 5$	Severe (AHI $>30$ ) vs controls	↑	↑	MMP-9 positively correlated with AHI, ODI and negatively correlated with mean SpO <sub>2</sub> .
							Severe vs mild/moderate	N	↑	
							Mild/moderate (5 $\leq$ AHI $<30$ ) vs controls	↑	↑	
Kaditis et al. [78]	Case-control	106	Type 1	MMP-9	Plasma	AHI $\geq 1$	Moderate/severe (AHI $>5$ ) vs mild (1 $\leq$ AHI $<5$ ) vs control		N	Study in children.
Maeder et al. [75]	Cross- sectional	71	Type 1 or type 3	MMP-9	Plasma	AHI $> 5$	Moderate/severe (AHI $\geq 15$ ) vs mild/no (AHI $<15$ )		N	
Tamaki et al. [71]	Case-control	46	Type 1	MMP-9	Blood monocytes	AHI $\geq 10$	Severe (AHI $\geq 30$ ) vs controls		↑	
							Mild/moderate (AHI $<30$ ) vs controls		↑	

Tazaki et al. [72]	Case-control	62	Type 1	MMP-9	Serum	AHI $\geq 5$	Moderate/severe (AHI $\geq 20$ ) vs controls		↑	MMP-9 level and activity positively correlated with AHI and SpO <sub>2</sub> <90%.
							Moderate/severe vs mild (5<AHI<20)		↑	
							Mild vs controls		↑	
Volná et al. [76]	Cross-sectional	51	Type 3	MMP-2 MMP-9	Serum	AHI > 5	Severe vs moderate vs mild vs controls	N	N	MMP-9 level positively correlated with ODI and SpO <sub>2</sub> <90% and negatively correlated with mean SpO <sub>2</sub> .
Wang et al. [56]	Cohort retrospective	47	Type 3	MMP-9	Serum	AHI $\geq 5$	Moderate (15 $\leq$ AHI<30) vs mild (5 $\leq$ AHI<15)		↑	MMP-9 level positively correlated with AHI, ODI and mean atrial pressure.
							Severe (AHI>30) vs mild		↑	
Ye et al. [73]	Case-control	76	Type 1	MMP-9	Serum	AHI $\geq 5$	Moderate/severe (AHI $\geq 20$ ) vs controls		↑	MMP-9 level positively correlated with AHI and SpO <sub>2</sub> <90%.
							Moderate/severe vs mild (5 $\leq$ AHI<20)		↑	
							Mild vs controls		↑	

Legend: AHI, apnea-hypopnea index; CRP, C-reactive protein; IHC immunohistochemical analysis; MMP, matrix metalloproteinase; ODI, oxygen desaturation index; OSA, obstructive sleep apnea; RDI, respiratory disturbance index; SpO<sub>2</sub>, blood oxygen saturation; SpO<sub>2</sub> <90%, time of blood oxygen saturation below 90%; \*according to American Academy of Sleep Medicine criteria; \*\* MMP concentration, unless otherwise noted; ↑ increase; ↓ decrease; N no difference; # without statistical significance

## **4 METHODOLOGY**

### **4.1 Data sources**

The study is a part of a large multi-center Canadian trial currently performed through the Canadian Sleep and Circadian Network (CSCN), which started data collection in 2016. Subjects were recruited among patients referred to the Sleep Disorders Center (Saskatoon City Hospital, Saskatchewan, Canada) for polysomnography (PSG). Written consent for participation in the study was obtained. Subjects completed a detailed medical and sleep questionnaire, were assessed for OSA by in-lab PSG, and provided blood and urine samples. This study was approved by the University of Saskatchewan Ethics Committee.

The database was accessed through REDCap (Research Electronic Data Capture) (<https://redcap.usask.ca/redcap/>). The database includes detailed physiologic data from polysomnography (e.g. AHI, ODI – oxygen desaturation index), detailed demographic and phenotypic data (e.g. age, gender, body mass index), and medical history including co-existing medical disorders such as cardiovascular diseases among others.

### **4.2 Materials**

Blood and urine samples were collected from all patients in the morning after in-lab PSG. Collection and treatment of blood samples have been performed according to SOP (Packet B) of the Center for Advanced Research in Sleep Medicine (CARSM) Sleep Research Biobank. In order to get serum, the Z Serum Clot Activator Sep tube (Greiner bio-one Vacuette Ref # 456018 or the equivalent) was used. The collection tube contains a clot activator (silicone dioxide) and a gel barrier in the base of the tube which separate the serum and the blood cells during centrifugation. After collection, the tubes were kept on a rack at room temperature for about 30 minutes (before centrifuging) to allow clotting of blood. Later on, the tubes were centrifuged at 3000 RPM (rotations per minute) for 10 minutes at room temperature. Next, serum was collected, aliquoted and frozen at - 80°C for later analysis.

Urine samples were centrifuged at 4000 RPM for 10 minutes at 4°C, collected without sediment, aliquoted and frozen at - 80°C.

### 4.3 Study groups

Subjects with and without OSA have been included in this study. All subjects underwent polysomnography, and were divided according to OSA severity as per American Academy of Sleep Medicine criteria [26]. Severity was established based on apnea/hypopnea index (AHI): number of events (apneas + hypopneas) per hour of sleep. *Apnea* was defined as a cessation of breathing for a minimum of 10 seconds. *Hypopnea* was defined as a decrease of airflow for more than 10 seconds associated with oxygen desaturation of 3% or an arousal.

Based on the above definitions the following study groups were defined:

1. Patients referred for PSG who were not diagnosed with OSA ( $AHI < 5$ ) – controls.
2. Patients with mild OSA ( $5 \leq AHI < 15$ )
3. Patients with moderate OSA ( $15 \leq AHI < 30$ )
4. Patients with severe OSA ( $AHI \geq 30$ )

Additionally, patients were divided according to cardiovascular status (with or without CVD). Cardiovascular comorbidities were herein defined as history of physician diagnosed arterial hypertension, coronary artery disease, heart failure, stroke and atrial fibrillation. Presence of these comorbidities has been verified by thorough medical records review.

### 4.4 Methods

#### 4.4.1 Polysomnography, anthropometrics and medical history

In-lab PSG was obtained using Sandman v.10, using the following channels: EEG, R and L EOG, chin EMG, airflow (pressure transducer and thermocouple), respiratory effort, anterior tibialis EMG, body position, EKG and oxygen saturation. PSG recordings were scored by a registered PSG technologist to the results of the study.

All patients self-completed a questionnaire which asked about socio-demographics and medical history, including a history of hypertension and physician diagnosed cardiovascular disease (coronary artery disease, heart failure, stroke, atrial fibrillation).

Body mass index (BMI) was calculated from height and weight measurements taken while patients were wearing light clothing.

#### 4.4.2 MMP-2 and MMP-9 activity assay (zymography)

Principles of the method: Gelatin zymography is a combination of two techniques:

1. Protein resolution in electric field.
2. Detection of protein and its measurement.

Samples were mixed with non-reducing sample loading buffer (in a ratio 3:1 (v/v)), and twenty  $\mu$ l of such prepared samples were applied to 8% polyacrylamide gels copolymerized with 2 mg/mL gelatin. The composition of sample loading buffer was: 0.5 M Tris-HCl containing 0,4% SDS (pH 6.8) - 70% (v/v), glycerol - 30% (v/v), SDS 10% - (w/v), bromophenol blue - 0.012% (w/v), ddH<sub>2</sub>O.

After electrophoresis (150V; 4°C), gels were rinsed in 2.5% Triton X-100 (3 times for 20 min) to remove SDS in order to gelatinases' renaturation. Then, the gels were washed in the incubation buffer for 10 min at room temperature. The composition of the incubation buffer was: 50 mM Tris-HCl pH 7.4, 5 mM CaCl<sub>2</sub>, 150 mM NaCl, 0.05% NaN<sub>3</sub>. The gels were incubated in the incubation buffer overnight at 37°C (24h for serum, and 48h for urine) in order to let the MMPs degrade gelatin embedded in the gel.

After incubation, gels were stained in staining solution (0.05% Coomassie Brilliant Blue R-250, 25% methanol, 10% acetic acid) for 2 h and then destained in destaining solution (4% methanol, 8% acetic acid). Gelatinolytic activities were observed as bright bands against the Coomassie stained background.

Zymograms were scanned using VersaDoc 5000 (BioRad), and the band intensities were analyzed by Quantity One software v. 4.6.6 (BioRad). MMP activities were expressed in arbitrary units (AU) as activity per 20  $\mu$ l of either serum or urine.

Internal standard was used for normalization of MMPs level. Purified human MMP-2 (MMP-2, active, human, recombinant, mouse cells, Millipore) was used as MMP-2 reference standard. To identify the MMPs based on their molecular weight, the molecular-weight size marker (Color Prestained Protein Standard, Broad Range, BioLabs,) was loaded to every gel.

The desired concentration of samples was established by performing gelatin zymography of different concentration of urine/serum samples and choosing the one that allows for detection of MMP activity. Urine samples were concentrated 10 times (Amicon Ultra 30K, Millipore). Serum samples were diluted 20 times with deionized water.



#### 4.4.3 Measurement of kidney function and level of kidney injury

##### 4.4.3.1 *Kidney function*

Renal function was measured with estimated glomerular filtration rate (eGFR). Glomerular filtration rate was estimated from serum creatinine, age, sex, and race using the CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration 2009) equation [92], recommended by The National Kidney Foundation.

The eGFR values greater than or equal to 90 mL/min/1.73 m<sup>2</sup> were considered as normal, 60-89 mL/min/1.73 m<sup>2</sup> as mildly decreased, 45-59 mL/min/1.73 m<sup>2</sup> as mildly to moderately decreased, 30-43 mL/min/1.73 m<sup>2</sup> as moderately to severely decreased, 15-29 mL/min/1.73 m<sup>2</sup> as severely decreased, and <15 mL/min/1.73 m<sup>2</sup> as kidney failure.

##### 4.4.3.2 *Glomerular injury*

Glomerular injury was measured by urinary albumin-to-creatinine ratio (ACR). The albuminuria categories were as followed: normal to mildly increased < 3 mg/mmol; moderately increased 3-30 mg/mmol; severely increased > 30 mg/mmol.

##### 4.4.3.3 *Tubular injury*

Neutrophil gelatinase-associated lipocalin (NGAL) – marker of tubular kidney injury – was measured with commercially available ELISA test (Lipocalin-2 (NGAL) Human ELISA KIT, Abcam, ab119600).

NGAL is considered useful in diagnosis of both acute kidney injury (AKI) and chronic kidney disease (CKD). Its levels correlate with severity of renal impairment, expressing the degree of tubular damage in response to different stress stimuli such as oxidative stress [93].

The assay was performed according to the manufacturer's instruction (Lipocalin-2 (NGAL) Human ELISA KIT, Abcam, ab119600). Briefly, urinary NGAL interacted with monoclonal antibodies specific for NGAL that have been coated onto the wells. Next, biotinylated polyclonal anti-NGAL antibodies were added, followed by their binding with ABC complex (Avidin-Biotin-Peroxidase Complex). The substrate solution was then used to visualize the HRP enzymatic reaction. The concentration of NGAL was proportional to the absorbance ( $\lambda = 450\text{nm}$ ) of the colored product.

#### 4.4.4 Statistical analysis

Data analysis was performed using SPSS version 25. Analyses were two-sided with an alpha of 0.05. Descriptive statistics are presented as mean  $\pm$  SD (standard deviation) and/or median with interquartile range (IQR) for continuous variables; and frequencies and proportions for categorical variables.

Objective 1: For this analysis, the primary independent variable was presence or absence of OSA. The dependent variables were level of MMP-2 and MMP-9. The independent-samples *t* test (if assumption of normality was met) or Mann-Whitney-Wilcoxon test (if data set was not normally distributed) was performed in order to compare two groups.

Objective 2: The primary independent variable was severity of OSA. The dependent variables were level of MMP-2 and MMP-9. ANOVA or the non-parametric equivalent (Kruskal-Wallis test) was used to compare more than two independent groups. In order to adjust for potential confounders, multiple linear regression was performed. The univariable analysis was conducted to determine the candidate variables for the multivariable models. This included assessment of correlation between MMP levels and apnea/hypopnea index using the Pearson correlation coefficient. Variables with *p*-value < 0.25 along with other potentially clinically important covariates were selected for the multiple linear regression. Log transformation of variables was used if the assumptions were not met.

Objective 3: For this analysis, only those participants with OSA were included. The primary independent variable was presence of cardiovascular comorbidity and the dependent variables were MMP-2 and MMP-9. The independent-samples *t* test (if assumption of normality was met) or Mann-Whitney-Wilcoxon test (if data set was not normally distributed) was performed in order to compare two groups.

Finally, kidney function and kidney injury were assessed to help explain potential associations observed around urinary MMP activities. The primary independent variable was severity of OSA, and the dependent variables were eGFR, ACR and NGAL. ANOVA or the non-parametric equivalent (Kruskal-Wallis test) was used to compare more than two independent groups. Then, urinary MMP-2-to-creatinine was compared between groups with normal to mildly increased albuminuria and moderately to severely increased albuminuria. The Mann-Whitney-Wilcoxon test was performed in order to compare these two groups. In order to adjust for potential confounders,

multiple linear regression was performed. The univariable analysis was conducted to determine the candidate variables for the multivariable models. Variables with p-value  $< 0.25$  along with other potentially clinically important covariates were selected for the multiple linear regression. Log transformation of variables was used if the assumptions were not met.

## 5 RESULTS

### 5.1 Comparisons of adult OSA patients and controls

A total of 150 patients were studied. Their demographic, anthropometric, and polysomnographic characteristics, stratified by OSA status, are presented in Table 5.1. There was no difference in the sex distribution between OSA and control patients (58.1% and 46.2% were males, respectively). Patients with OSA were older ( $56.3 \pm 12.0$  vs  $47.2 \pm 12.5$  years;  $p=0.001$ ) and had significantly higher weight ( $101.3 \pm 26.9$  vs  $83.5 \pm 15.4$  kg;  $p=0.002$ ) and body mass index ( $34.2 \pm 7.7$  vs  $28.4 \pm 4.5$  kg/m<sup>2</sup>;  $p<0.001$ ) compared to control patients. By definition, AHI was higher in patients with OSA than in control patients ( $33.4 \pm 29.2$  vs  $2.8 \pm 1.4$  events/h). The prevalence of CVD was significantly higher in OSA compared to control patients (54.0% vs 19.2%;  $p=0.001$ ).

MMP activities are presented in Table 5.2. Serum MMP-2 activity was significantly higher in patients with OSA than in controls ( $p = 0.029$ ; Figure 5.1A).

Although urinary MMP-2 activity in OSA patients was higher than in controls, the difference was not statistically significant ( $p = 0.129$ ; Figure 5.1B).

Mean activities of MMP-9 for both serum and urine were higher in OSA patients compared to control, however the differences were not shown to be statistically significant (Figure 5.1C-D).

Table 5.1 Baseline characteristics of subjects stratified by OSA status.

	Control (N = 26)	OSA (N = 124)	p-value
Sex: male	12 (46.2%)	72 (58.1%)	0.266
Age (years)	47.2 ± 12.5	56.3 ± 12.0	<b>0.001</b>
Weight (kg)	83.5 ± 15.4	101.3 ± 26.9	<b>0.002</b>
Height (m)	171.40 ± 8.74	171.9 ± 9.44	0.820
BMI (kg/m <sup>2</sup> )	28.4 ± 4.5	34.2 ± 7.7	<b>&lt;0.001</b>
AHI (n/hr)	2.8 ± 1.4	33.4 ± 29.2	<b>&lt; 0.001</b>
ODI (n/hr)	3.97 ± 3.67	35.2 ± 33.4	<b>&lt; 0.001</b>
CVD occurrence: yes	5 (19.2%)	67 (54%)	<b>0.001</b>
Type 2 diabetes: yes	1 (3.8%)	19 (15.3%)	0.201

Data are presented as mean ± SD or frequencies (%). AHI, apnea/hypopnea index per hour of sleep; BMI, body mass index; CVD defined as: arterial hypertension, coronary artery disease, heart failure, stroke and atrial fibrillation; ODI, oxygen desaturation index (3%) per hour of sleep.

Comparisons were performed by using the independent-samples *t* test or Chi-square test.

Table 5.2 Comparison of MMPs activity between patients with OSA and Controls.

	Control	OSA	p value
	median (25th–75th percentile)		
	mean (95% CI)		
MMP-2 serum	134.9 (92.7 – 185.7) 143.1 (115.8 – 170.4)	176.2 (104.7 – 298.2) 304.7 (210.4 – 399.0)	<b>0.029</b>
MMP-2 urine	0.037 (0.005 – 0.117) 0.106 (0.040 – 0.172)	0.075 (0.010 – 0.320) 0.284 (0.189 – 0.378)	0.129
MMP-9 serum	1100 (240 – 3182) 1800 (1037 – 2564)	928 (430 – 2287) 2381 (1533 – 3229)	0.783
MMP-9 urine	0.11 (0.00 – 0.70) 1.93 (0.00 – 4.24)	0.36 (0.00 – 3.40) 7.37 (4.43 – 10.30)	0.084

Comparisons were performed by using the Mann-Whitney-Wilcoxon test.

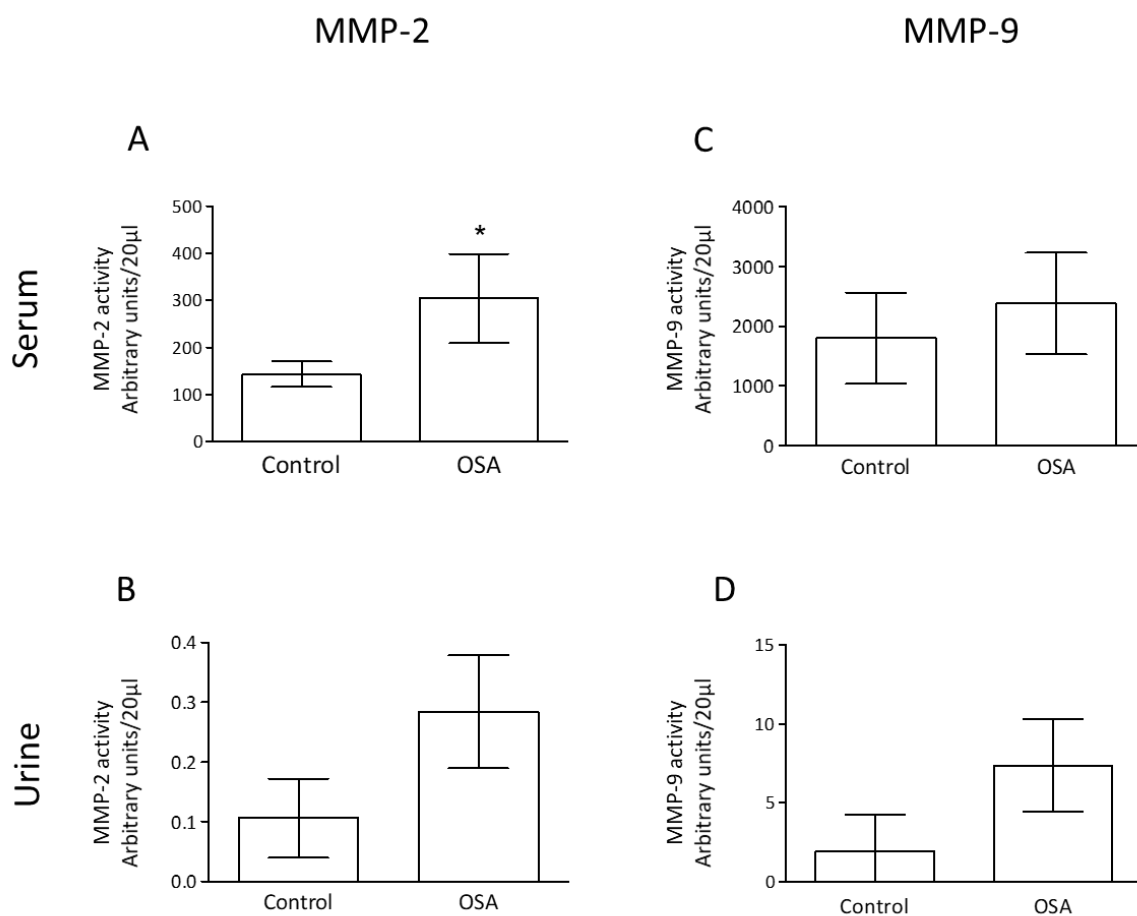


Figure 5.1 MMP activities in patient with OSA and controls.

Comparison of MMP activities between patients with and without OSA: serum MMP-2 (A), urinary MMP-2 (B), serum MMP-9 (C), urinary MMP-9 (D). OSA – obstructive sleep apnea; \* $p < 0.05$  vs control; mean, error bars: 95% confidence interval.

## **5.2 Comparisons in relation to the severity of OSA**

Characteristics of subjects, stratified by OSA severity, are shown in Table 5.3. There were no differences in sex and height distribution between the groups. The patients with more severe OSA tended to be heavier (weight, BMI) than those with less severe disease or the controls. There was a significant difference in prevalence of CVD among the groups. Severe OSA cases had high prevalence of CVD (63.8%), in both mild and moderate OSA groups the prevalence of CVD was approximately 48%, and in control group it was only 19.25% ( $p = 0.004$ ).

MMP activities in relation to OSA severity differ for MMP-2 in serum, and MMP-9 in both serum and urine (Figure 5.2A-D, Table 5.4).

Table 5.3 Characteristics of subjects stratified by OSA categories.

	Control (N = 26)	Obstructive sleep apnea			p- value
		Mild (N = 43)	Moderate (N = 34)	Severe (N = 47)	
Sex: male	12 (46.2%)	20 (46.5%)	21 (61.8%)	31 (66.0%)	0.176
Age (years)	47.2 ± 12.5	57.7 ± 12.0 *	55.6 ± 12.5 *	55.5 ± 11.8 *	<b>0.006</b>
Weight (kg)	83.6 ± 15.4	91.7 ± 15.9	96.9 ± 19.6	113.1 ± 34.4 *#§	< <b>0.001</b>
Height (m)	171.4 ± 8.7	171.1 ± 8.8	171.6 ± 10.4	172.7 ± 9.4	0.869
BMI (kg/m <sup>2</sup> )	28.4 ± 4.5	31.3 ± 5.1	32.9 ± 5.8	37.7 ± 9.5 *#§	< <b>0.001</b>
AHI (n/hr)	2.8 ± 1.4	9.3 ± 2.9	22.0 ± 4.6	63.7 ± 26.1	< <b>0.001</b>
ODI (n/hr)	3.97 ± 3.67	10.36 ± 4.35	23.03 ± 7.30	66.74 ± 34.98	< <b>0.001</b>
CVD occurrence: yes	5 (19.2%)	21 (48.8%)	16 (47.1%)	30 (63.8%)	<b>0.004</b>
Hypertension: yes	5 (19.2%)	15 (34.9%)	13 (38.2%)	28 (59.6%)	<b>0.006</b>
Diabetes type 2: yes	1 (3.8%)	4 (9.3%)	5 (14.7%)	10 (21.3%)	0.170

Data are presented as mean ± SD or frequencies (%). \* p<0.05 vs control group; # p<0.05 vs mild OSA; § p<0.05 vs moderate OSA. BMI, body mass index; CVD defined as: arterial hypertension, coronary artery disease, heart failure, stroke and atrial fibrillation.

Comparisons were performed by using one-way ANOVA or Chi-square test.



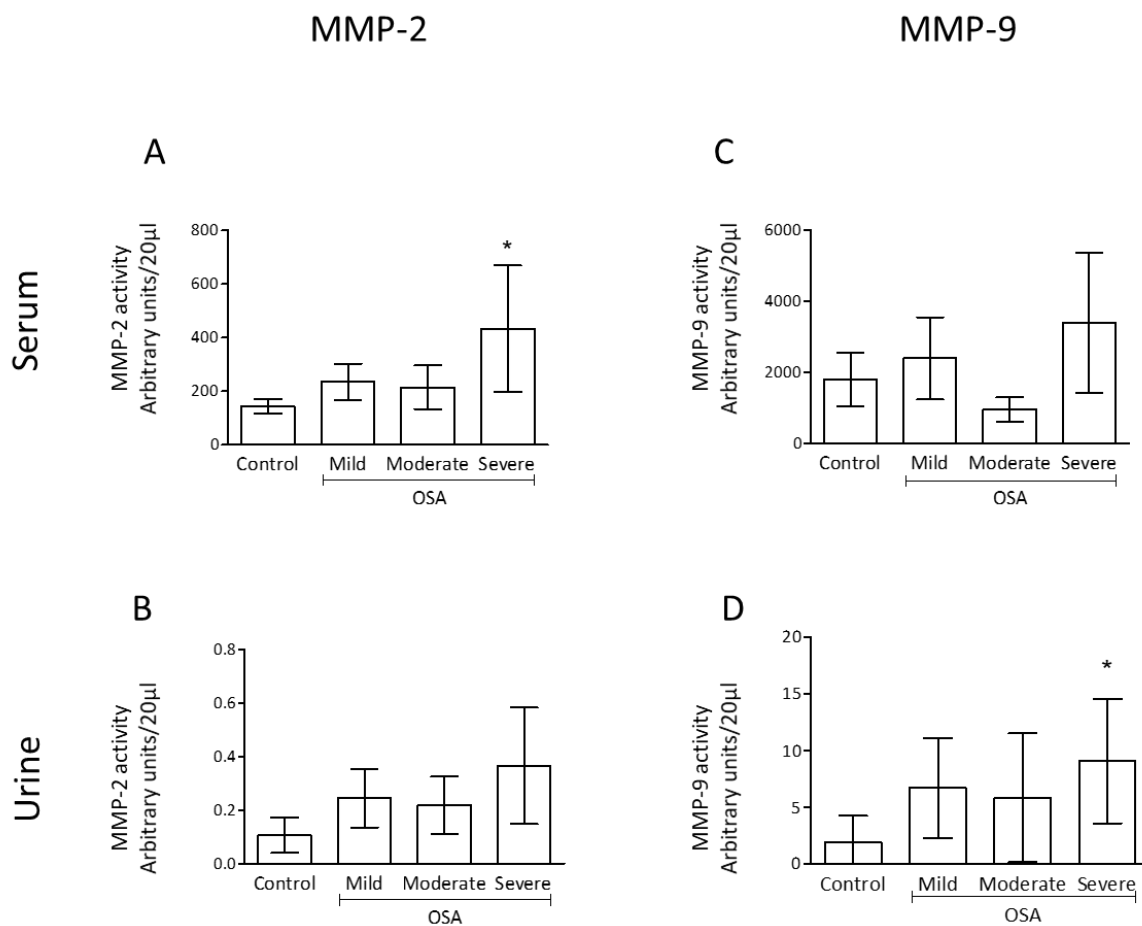


Figure 5.2 MMP activities in regards to OSA severity.

Comparisons for serum MMP-2 (A), urinary MMP-2 (B), serum MMP-9 (C), urinary MMP-9 (D).

OSA – obstructive sleep apnea; \*p < 0.05 vs control; mean, error bars: 95% confidence interval.

Table 5.4 MMPs activity according to OSA severity.

	Non-OSA (N = 26)	Mild (N = 43)	Moderate (N = 34)	Severe (N = 47)	p-value
		Median (25th–75th percentile) Mean (95% CI)			
MMP-2 serum	135 (93 – 186) 143 (116 – 170)	180 (100 – 282) 235 (167 – 303)	133 (90 – 198) 215 (133 – 297)	229 (127 – 368) * § 434 (198 – 669)	<b>0.003</b>
MMP-2 urine	0.04 (0.01 – 0.12) 0.11 (0.04 – 0.17)	0.10 (0.01 – 0.32) 0.24 (0.14 – 0.35)	0.10 (0.02 – 0.34) 0.22 (0.11 – 0.33)	0.06 (0.01 – 0.28) 0.37 (0.15 – 0.58)	0.475
MMP-9 serum	1100 (240 – 3182) 1800 (1037 – 2564)	1230 (594 – 2316) 2400 (1249 – 3551)	573 (211 – 1510) # 951 (605 – 1297)	950 (540 – 2480) § 3398 (1431 – 5364)	<b>0.039</b>
MMP-9 urine	0.11 (0.00 – 0.70) 1.93 (0.00 – 4.24)	0.390(0.00 – 5.05) 6.70 (2.31 – 11.09)	0.05 (0.00 – 1.50) 5.84 (0.18 – 11.51)	0.84 (0.04 – 3.75) * § 9.04 (3.55 – 14.53)	<b>0.028</b>

\*p<0.05 vs control group; # p<0.05 vs mild OSA; § p<0.05 vs moderate OSA. Comparisons were performed by using Kruskal-Wallis test.

### 5.2.1 MMP-2 activity

The highest serum MMP-2 activity was observed in patients with severe OSA. Serum MMP-2 activity in patients with severe OSA was significantly higher than in those with moderate OSA and controls (Figure 5.2A, Table 5.4).

Simple linear regression showed statistically significant associations with MMP-2 activity in serum for AHI ( $p = 0.001$ ; Figure 5.3A), ODI ( $p = 0.004$ ; Figure 5.3B), and age ( $p = 0.023$ ) (Table 5.5). The association between MMP-2 and AHI (Model 1) as well as MMP-2 and ODI (Model 2) persisted even after adjustment for age, sex, BMI, and CVD (Table 5.6).

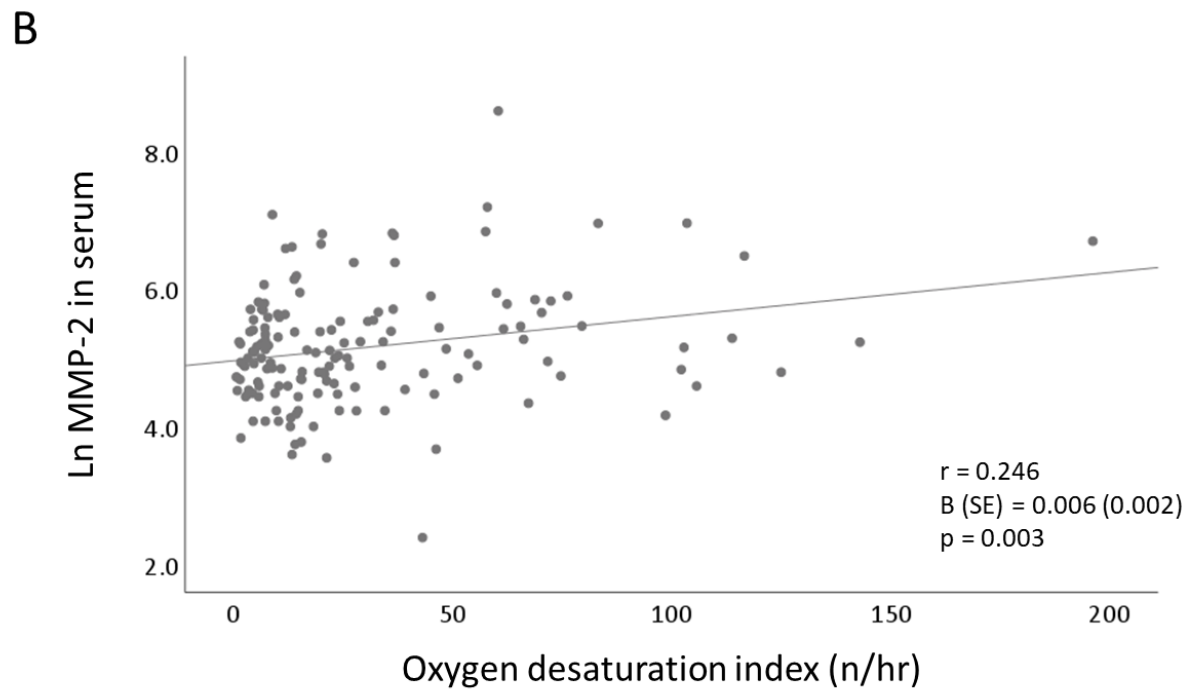
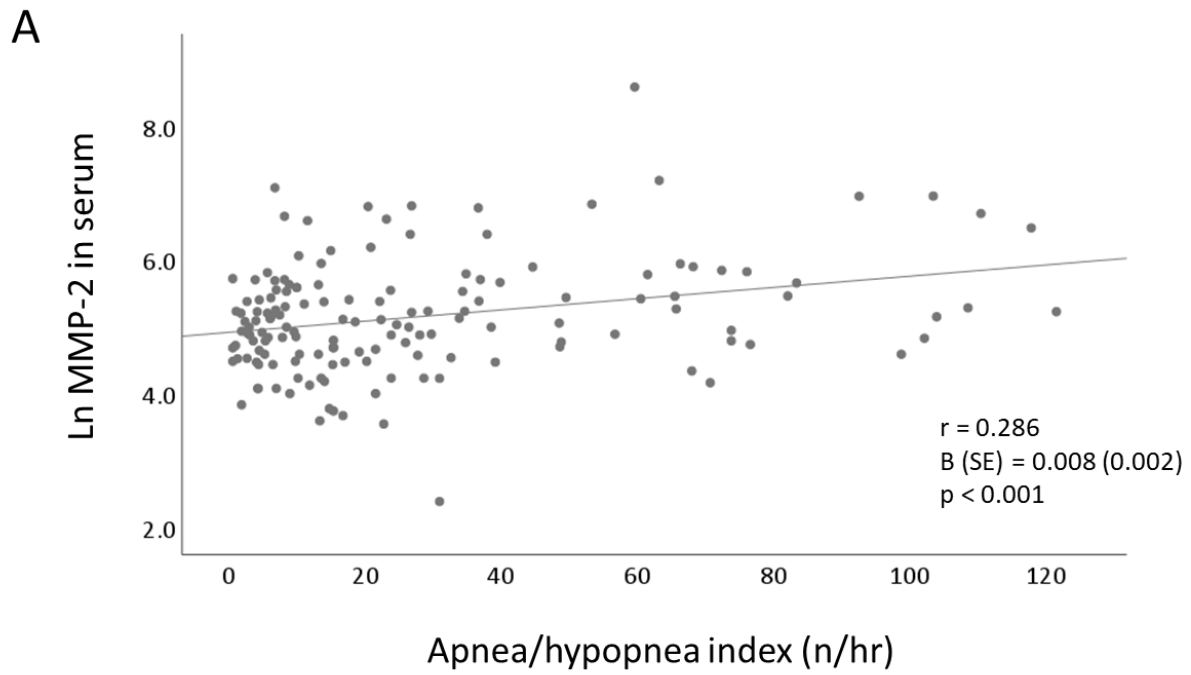


Figure 5.3 Linear regression of ln MMP-2 and AHI (A) and ODI (B) in study subjects.

Table 5.5 Crude linear regression analysis of ln MMP-2 activity in serum on risk factors and other potentially important covariates.

Variable	r	Parameter estimate (SE)	p-value
<b>AHI</b>	0.286	0.008 (0.002)	<b>&lt; 0.001</b>
<b>ODI</b>	0.246	0.006 (0.002)	<b>0.003</b>
<b>Age</b>	0.203	0.014 (0.005)	<b>0.013</b>
<b>Sex</b> (reference is male) Female	0.088	-0.150 (0.140)	0.283
<b>BMI</b>	0.122	0.014 (0.009)	0.138
<b>CVD</b> (reference is No) Yes	0.148	0.250 (0.138)	0.072
<b>WBC</b>	0.003	-0.001 (0.039)	0.973

Table 5.6 Results from multiple linear regression analysis with serum MMP-2 as the outcome.

Variable	Model 1		Model 2	
	Parameter estimate (SE)	p-value	Parameter estimate (SE)	p-value
<b>Risk factor of primary interest</b>				
AHI	0.008 (0.003)	<b>0.004</b>		
ODI			0.006 (0.002)	<b>0.017</b>
<b>Age</b>	0.012 (0.006)	<b>0.046</b>	0.012 (0.006)	<b>0.034</b>
<b>Sex</b> (reference is male) Female	-0.087 (0.135)	0.519	-0.122 (0.136)	0.368
<b>BMI</b>	0.001 (0.010)	0.958	0.003 (.010)	0.778
<b>CVD</b>	0.083 (0.146)	0.570	0.082 (0.147)	0.580

### 5.2.2 MMP-9 activity

MMP-9 activity in serum ( $p = 0.039$ ) and MMP-9 activity in urine ( $p = 0.028$ ) differed significantly between groups. MMP-9 activity in serum was significantly lower in patients with moderate OSA compared to severe OSA and mild OSA.

Simple linear regression showed statistically significant associations with MMP-9 activity in serum for AHI ( $p = 0.043$ ), ODI ( $p = 0.027$ ), white blood cells (WBC) count ( $p < 0.001$ ; Figure 5.4) and age ( $p = 0.049$ ) (Table 5.7). Serum MMP-9 activity was associated with WBC count, independently of AHI/ODI, age, sex, BMI and CVD (Table 5.8).

In urine, MMP-9 activity was significantly higher in severe OSA patients than in moderate OSA patients and controls.

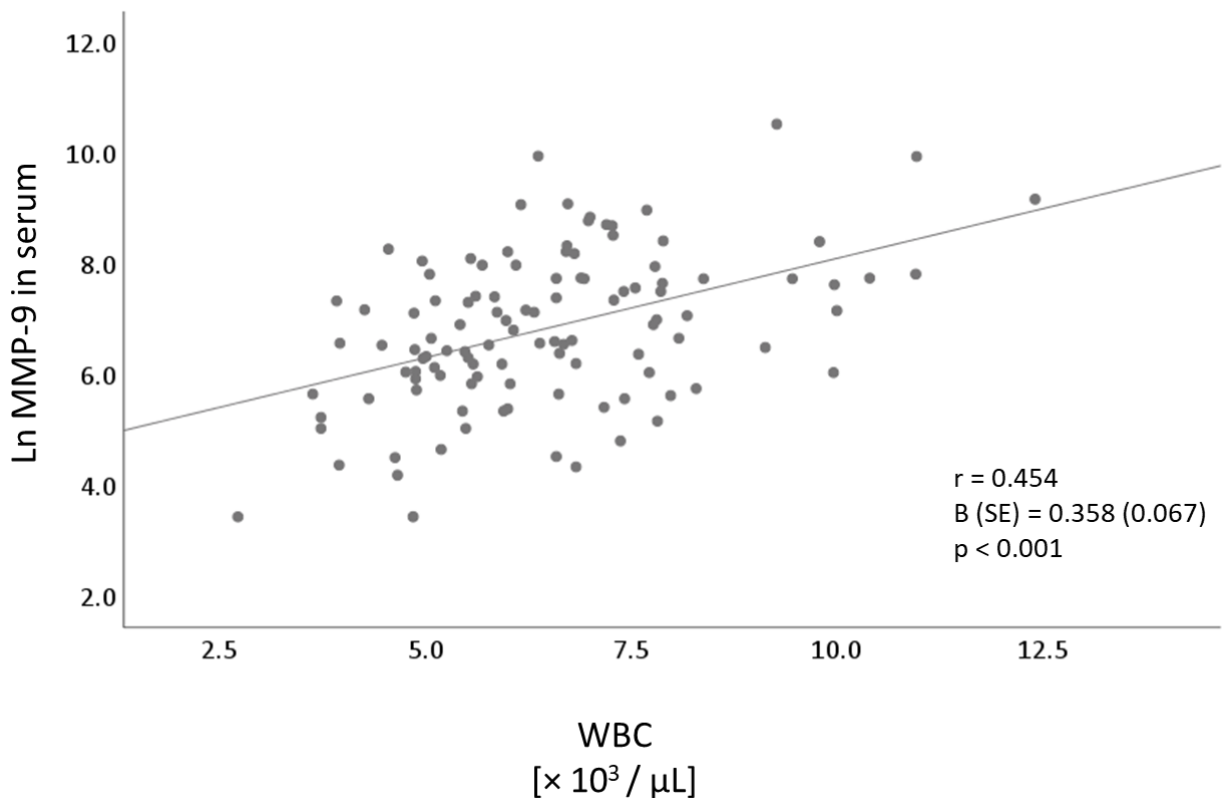


Figure 5.4 Linear regression of ln MMP-9 and WBC (white blood cell) count.

Table 5.7 Crude linear regression analysis of ln MMP-9 activity in serum on risk factors and other potentially important covariates.

Variable	r	Parameter estimate (SE)	p-value
<b>AHI</b>	0.165	0.008 (0.004)	<b>0.043</b>
<b>ODI</b>	0.181	0.007 (0.003)	<b>0.027</b>
<b>WBC</b>	0.454	0.358 (0.067)	<b>&lt; 0.001</b>
<b>Age</b>	0.161	-0.017 (0.009)	<b>0.049</b>
<b>Sex</b> (reference is male)			
Female	0.037	-0.101 (0.223)	0.652
<b>BMI</b>	0.183	0.033 (0.014)	0.025
<b>CVD</b> (reference is No)	0.012	0.033 (0.221)	0.881
Yes			

Table 5.8 Results from multiple linear regression analysis with ln serum MMP-9 as the outcome.

Variable	Model 1		Model 2	
	Parameter estimate (SE)	p-value	Parameter estimate (SE)	p-value
<b>WBC</b>	0.310 (0.079)	<b>&lt; 0.001</b>	0.300 (0.081)	<b>&lt; 0.001</b>
<b>AHI</b>	0.008 (0.005)	0.132		
<b>ODI</b>			0.005 (0.004)	0.226
<b>Age</b>	-0.014 (0.011)	0.191	-0.013 (0.011)	0.215
<b>Sex</b> (reference is male)				
Female	-0.235 (0.238)	0.327	-0.264 (0.238)	0.270
<b>BMI</b>	-0.011 (0.023)	0.631	-0.005 (0.024)	0.851
<b>CVD</b> (reference is No)				
Yes	0.018 (0.262)	0.944	0.021 (0.264)	0.937

### 5.3 Comparisons in regards to cardiovascular disease occurrence

OSA patients with and without CVD did not differ in regards to sex distribution, weight, height, BMI, and polysomnographic parameters (Table 5.9). The groups differed only in regards to age ( $52.2 \pm 10.8$  vs  $59.7 \pm 12.0$  for Non-CVD and CVD, respectively).

There were no differences in MMP-2 and MMP-9 activities between patients with and without CVD (Table 5.10, Figure 5.5A-D).

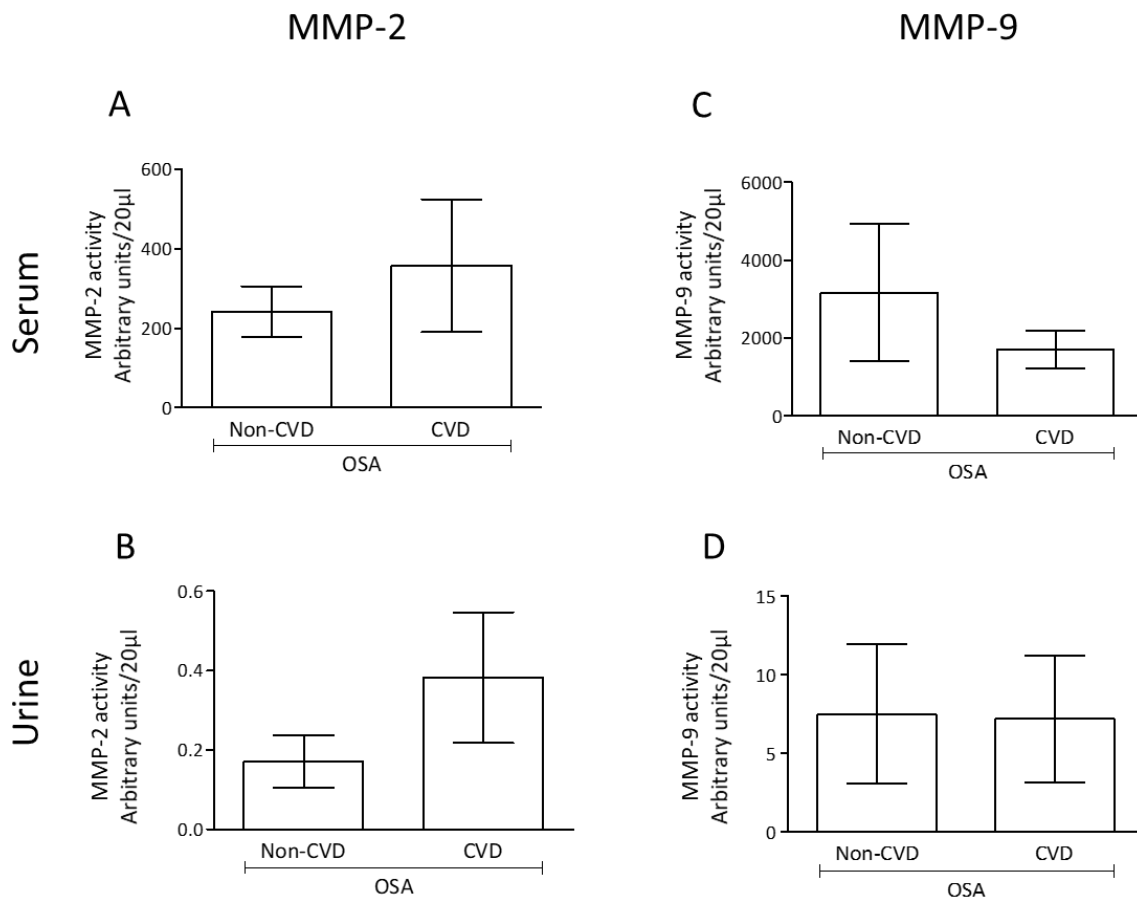


Figure 5.5 MMP activities in regards to cardiovascular disease occurrence.

Comparisons for serum MMP-2 (A), urinary MMP-2 (B), serum MMP-9 (C), urinary MMP-9 (D). OSA – obstructive sleep apnea; CVD – cardiovascular disease; \* $p < 0.05$  vs Non-CVD; mean, error bars: 95% confidence interval.



Table 5.9 Baseline characteristics of subjects stratified by CVD status.

	Non-CVD (N = 57)	CVD (N = 67)	p-value
Sex: male	32 (56.1%)	40 (59.7%)	0.689
Age (years)	52.2 ± 10.8	59.7 ± 12.0	<b>&lt; 0.001</b>
Weight (kg)	98.5 ± 24.9	103.6 ± 28.4	0.299
Height (m)	172.5 ± 10.1	171.3 ± 8.9	0.489
BMI (kg/m <sup>2</sup> )	32.9 ± 6.9	35.2 ± 8.3	0.094
AHI (n/hr)	32.3 ± 31.3	34.6 ± 27.6	0.692
ODI (n/hr)	33.1 ± 32.5	37.0 ± 34.3	0.513

Data are presented as mean ± SD or frequencies (%). AHI, apnea/hypopnea index per hour of sleep; BMI, body mass index; CVD defined as: arterial hypertension, coronary artery disease, heart failure, stroke and atrial fibrillation; ODI, oxygen desaturation index (3%) per hour of sleep.

Comparisons were performed by using the independent-samples *t* test or Chi-square test

Table 5.10 MMP activity in OSA patients with and without cardiovascular disease (CVD).

	OSA		p-value
	Non-CVD	CVD	
	median (25th–75th percentile) mean (95% CI)		
MMP-2 serum	167 (89 – 277) 242 (178 – 306)	185 (120 – 332) 358 (191 – 525)	0.176
MMP-2 urine	0.06 (0.01 – 0.26) 0.17 (0.10 – 0.24)	0.12 (0.01 – 0.47) 0.38 (0.21 – 0.55)	0.239
MMP-9 serum	950 (458 – 2288) 3174 (1406 – 4942)	905 (421 – 2286) 1706 (1232 – 2180)	0.944
MMP-9 urine	0.35 (0.01 – 3.02) 7.52 (3.10 – 11.9)	0.47 (0.00 – 3.98) 7.23 (3.20 – 11.26)	0.804

Comparisons were performed by using the Mann-Whitney-Wilcoxon test.

Serum MMP-2 activity differed between groups at different OSA severity in patients with CVD ( $p = 0.029$ ), not in those without CVD ( $p = 0.217$ ). Severe OSA patients with CVD had significantly higher serum MMP-2 level compared to the controls; which was not observed in patients without CVD (Figure 5.6).

The associations found between MMP-2 and AHI (Model 1) as well as MMP-2 and ODI (Model 2) (Table 5.6; Figure 5.3), when stratified by CVD, remained significant only for patients with CVD, not for those without CVD (Table 5.11).

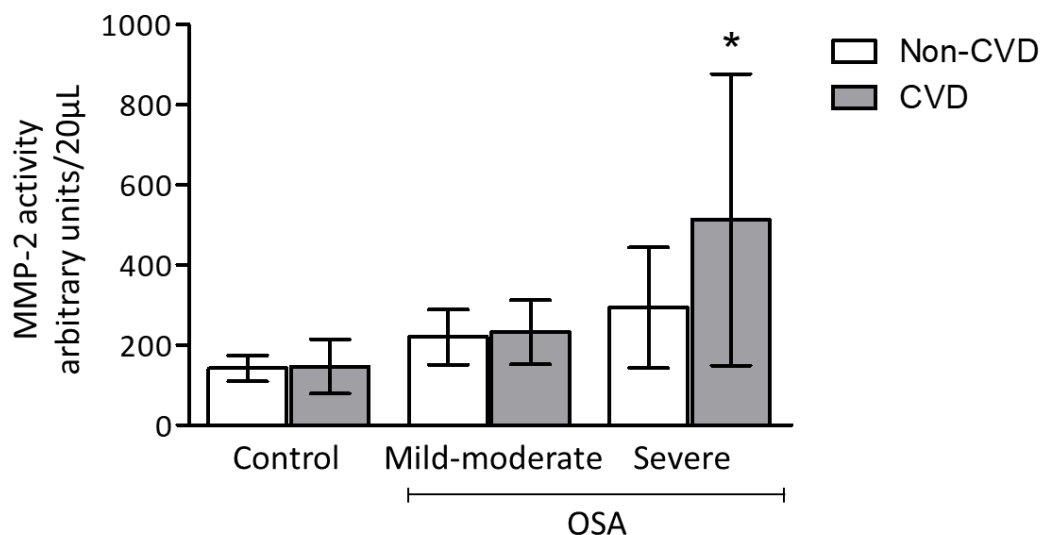


Figure 5.6 Serum MMP-2 activity in regards to OSA severity and CVD status.

Mild and moderate groups were merged as there were no differences between them in regards to their demographic and anthropometric characteristics (Table 5.3), and serum MMP-2 level (Table 5.4, Figure 5.2).

\* $p < 0.05$  vs control; mean, error bars: 95% confidence interval.

Table 5.11 Results from multiple linear regression analysis with ln MMP-2 in serum as the outcome, stratified by CVD.

Variable	Model 1				Model 2			
	Non-CVD		CVD		Non-CVD		CVD	
	Parameter estimate (SE)	p-value	Parameter estimate (SE)	p-value	Parameter estimate (SE)	p-value	Parameter estimate (SE)	p-value
<b>Risk factor of primary interest</b>								
AHI	0.006 (0.004)	0.084	0.011 (0.004)	<b>0.009</b>				
ODI					0.005 (0.004)	0.188	0.007 (0.003)	<b>0.030</b>
<b>Age</b>	0.007 (0.008)	0.347	0.017 (0.009)	<b>0.049</b>	0.008 (0.008)	0.340	0.019 (0.009)	<b>0.037</b>
<b>Sex</b> (reference is male)								
Female	-0.185 (0.175)	0.294	0.014 (0.211)	0.948	-0.195 (0.177)	0.274	-0.059 (0.213)	0.783
<b>BMI</b>	-0.014 (0.016)	0.388	0.009 (0.014)	0.513	-0.009 (0.017)	0.575	0.011 (0.014)	0.454

#### 5.4 Kidney function and level of kidney injury

There was no difference in eGFR between groups (Figure 5.7A, Table 5.12). The mean eGFR values for severe OSA patients were within the normal range  $91.6 \pm 16.7$  ml/min/1.73m<sup>2</sup>). Mildly decreased eGFR was observed for control ( $88.6 \pm 12.2$  ml/min/1.73m<sup>2</sup>), mild OSA ( $87.6 \pm 14.0$  ml/min/1.73m<sup>2</sup>) and moderate OSA ( $83.6 \pm 30.5$  ml/min/1.73m<sup>2</sup>).

The NGAL level did not differ between groups (Figure 5.7C, Table 5.12). There was no significant difference in mean ACR level between groups (Figure 5.7B, Table 5.12). However, the more severe sleep apnea, the higher ACR mean values. ACR means for control, mild and moderate OSA were within the range of category of normal to mildly increase albuminuria ( $1.0 \pm 2.1$ ;  $1.2 \pm 2.1$ ;  $1.9 \pm 4.9$  mg/mmol, respectively), severe OSA patients had moderately increased albuminuria ( $6.7 \pm 7.0$  mg/mmol).

Table 5.12 Kidney function and kidney injury in study patients.

	Control	Obstructive sleep apnea			p-value
		Mild	Moderate	Severe	
eGFR	$88.6 \pm 12.2$	$87.6 \pm 14.0$	$83.6 \pm 30.5$	$91.6 \pm 16.7$	0.476
ACR	$1.0 \pm 2.1$	$1.2 \pm 2.1$	$1.9 \pm 4.9$	$4.5 \pm 13.8$	0.211
NGAL	$6.0 \pm 6.2$	-	$7.5 \pm 9.8$	$6.7 \pm 7.0$	0.865

There was statistically significant difference in urinary MMP-2 activity (adjusted for creatinine – indicator of hydration status) in OSA patients stratified by level of albuminuria (normal to mildly increased vs moderately to severely increased) ( $p = 0.042$ , Figure 5.7D).

Simple linear regression showed statistically significant associations with urinary MMP-2 activity-to-creatinine for ACR ( $p = 0.020$ , Figure 5.8), serum MMP-2 ( $p = 0.016$ ), and hypertension ( $p = 0.022$ ) – see Table 5.13. The association between ACR and urinary MMP-2-to-creatinine persisted after adjustment for eGFR, serum MMP-2, age, sex, BMI, hypertension and type 2 diabetes (Table 5.14).

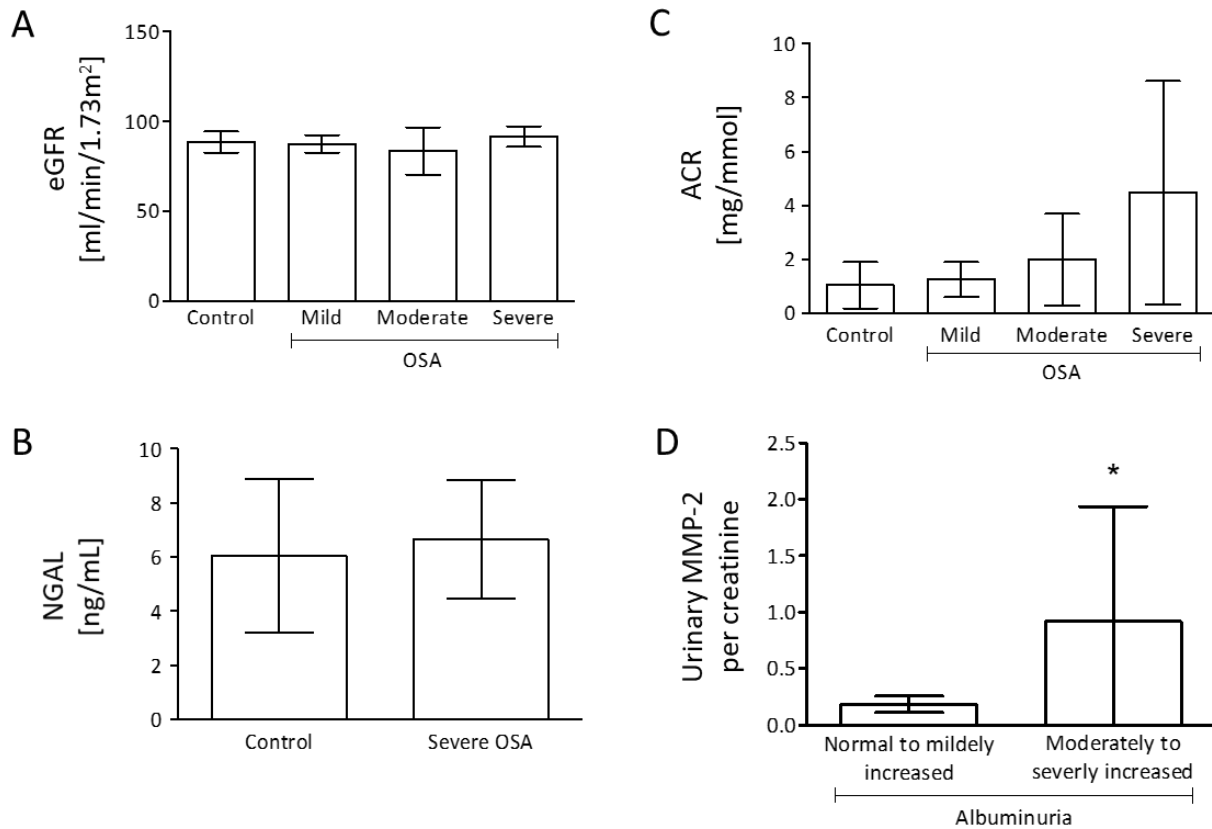


Figure 5.7 Level of kidney function and kidney injury in study subjects.

Level of kidney function measured by eGFR (estimated glomerular filtration rate) (A); level of kidney injury measured by NGAL (neutrophil gelatinase associated lipocalin) (B); level of glomerular injury measured by ACR (albumin-to-creatinine ratio) (C); urinary MMP-2 per creatinine in in OSA patients in regards to level of glomerular damage (D).

\* $p < 0.05$  vs another group; mean, error bars: 95% confidence interval.

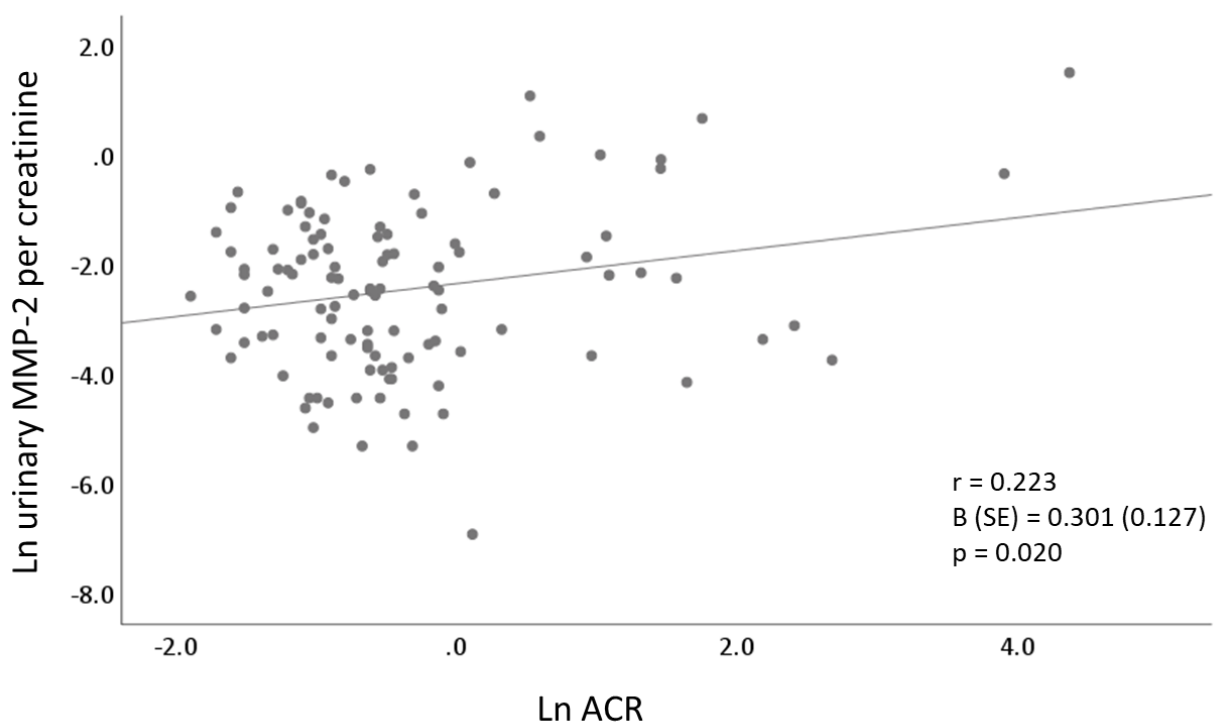


Figure 5.8 Linear regression of urinary MMP-2/creatinine and ACR in study subjects.

Table 5.13 Crude linear regression analysis of ln urinary MMP-2-to-creatinine on potentially important covariates.

Variable	r	Parameter estimate (SE)	p-value
<b>ACR</b>	0.223	0.301 (0.127)	<b>0.020</b>
<b>eGFR</b>	0.147	-0.012 (0.009)	<b>0.174</b>
<b>MMP-2 in serum</b>	0.219	0.380 (0.156)	<b>0.016</b>
<b>Age</b>	0.176	0.022 (0.011)	<b>0.055</b>
<b>Sex</b> (reference is male)			
Female	0.005	-0.014 (0.288)	0.961
<b>BMI</b>	0.001	0.000 (0.018)	0.995
<b>Type 2 diabetes</b> (reference is No)			
Yes	0.046	0.200 (0.401)	0.619
<b>Systemic hypertension</b>	0.209	0.660 (0.284)	<b>0.022</b>
Yes			
<b>AHI</b>	0.103	0.005 (0.005)	0.261

Table 5.14 Results from multiple linear regression analysis with urinary MMP-2-to-creatinine as the outcome.

Variable	Parameter estimate (SE)	p-value
<b>ACR</b>	0.406 (0.173)	<b>0.022</b>
<b>eGFR</b>	-0.012 (0.011)	0.256
<b>MMP-2 in serum</b>	0.270 (0.216)	0.217
<b>Age</b>	-0.002 (0.015)	0.888
<b>Sex</b> (reference is male)		
Female	-0.436 (0.333)	0.195
<b>BMI</b>	-0.013 (0.027)	0.621
<b>Type 2 diabetes</b> (reference is No)		
Yes	-0.618 (0.471)	0.194
<b>Systemic hypertension</b> (reference is No)	0.268 (0.358)	0.457
Yes		

## 6 DISCUSSION

Recurrent episodes of complete and/or partial obstruction of upper airways in OSA frequently result in a repetitive transient decrease in arterial oxygen saturation and may in turn lead to oxidative stress [10]. Intermittent hypoxia and arousals initiate the adverse physiological and biochemical sequelae which result in numerous adverse consequences including cardiovascular disease [9,94]. It has been shown that a 10-unit average increase in AHI was associated with a 17% greater risk of CVD in OSA [7]. Therefore, finding a simple non-invasive biomarker of severity of OSA and hypoxic burden is crucial, as it can help identify those OSA patients who may be at risk of adverse consequences related to OSA severity [8]. Different molecules (including vascular proteins, adhesion molecules, inflammatory markers, catecholamines and oxidative stress markers) have been explored as potentially useful prognostic biomarkers, however, the results are inconsistent [24]. There are plausible biological mechanisms, including oxidative stress and inflammation, linking MMPs and OSA (see Figure 3.1).

The main results of my thesis are as followed:

- Patients with OSA and without OSA significantly differ in regards to serum MMP-2 activity.
- Serum MMP-2 activity was significantly associated with OSA severity/level of hypoxemia in OSA, even after adjustment for sex, BMI and CVD.
- Serum MMP-9 activity was associated with white blood cells count, independently of AHI/ODI, age, sex, BMI and CVD.
- Urinary MMP-2 activity was associated with glomerular proteinuria (independently of hypertension and diabetes).
- There were no differences in MMP-2 and MMP-9 activities between patients with and without CVD, however, when stratified by CVD, the association between serum MMP-2 and AHI/ODI remain significant only for patients with CVD.

### 6.1 MMP-2 in serum

The previous evidence linking MMP-2 and OSA severity is scarce. A systematic review [15] revealed only three papers concerning circulating MMP-2 in adults with OSA [70,74,76].



Among them, one study [70] reported an increased MMP-2 plasma level in OSA patients, another [76] showed no association between OSA and serum levels of MMP-2, and the last one [74] reported that MMP-2 level in serum was lower in patients with severe OSA compared to subjects with less severe sleep apnea.

The results of our study demonstrate higher activity of serum MMP-2 in OSA than in controls (Figure 5.1). The results can be explained by the fact that MMP-2 is synthesized as an inactive zymogen, and can be activated by ROS [45], which are produced during CIH [10,95]. The results are also in accordance with a study on human endothelial cell culture which showed that hypoxia significantly enhanced MMP-2 activity [57].

Moreover, it was shown in a rat model that acute ischemia/reperfusion injury leads to marked activation of MMP-2, which was positively associated with duration of ischemia [44]. The severity of OSA is associated with greater number of apnea/hypopnea events and thus oxidative stress increases along with OSA severity (there was a strong correlation between AHI and ODI in our cohort;  $r = 0.952$ ,  $\beta(\text{SE}) = 1.072 (0.028)$ ,  $p < 0.001$ ). Therefore, the positive associations between serum MMP-2 and AHI as well as MMP-2 and particularly ODI, which were found in this study, are not surprising (Table 5.6). The inconsistency with the previous findings can result from insufficient statistical power because of low sample sizes (ranging between 50-79). Also, using RDI for quantification of OSA severity, as was done previously, does not properly reflect the hypoxemic insult in OSA patients, and underestimation of desaturations could be even greater than in case of using AHI. It is possible that patients with less severe OSA ( $\text{RDI} < 30$ ) experienced higher desaturations (higher oxidative stress) than severe OSA patients ( $\text{RDI} > 30$ ), and it could be an explanation for results observed by Bonanno et al. [74] who reported lower serum MMP-2 level in patients with severe OSA ( $\text{RDI} > 30$ ) compared to subjects with less severe OSA ( $\text{RDI} < 30$ ). However, due to limited data in study of Bonanno et al. we cannot assess if this reasoning could be confirmed.

MMP-2 has been previously shown to be associated with CVD, and interestingly, when results were stratified by CVD status, the association between serum MMP-2 activity and AHI/ODI remained significant only for patients with CVD (Table 5.11). However, the interaction between OSA severity and CVD cannot be fully assessed, as the study might not have enough statistical power.

OSA exposes cardiovascular system to various noxious stimuli (oxidative stress, inflammation, endothelial dysfunction, autonomic dysregulation, negative intrathoracic pressure) [96]. All of these can mediate the development or progression of CVD. While in our study CVD was a composite measure, in future larger studies MMP-2 should be thoroughly investigated in the subgroups of patients with different CVDs.

Increased MMP levels has been shown in clinical and experimental models of systemic hypertension [97]. Apart from ROS and inflammatory cytokines, MMP-2 can be activated by angiotensinogen II [98], which is frequently elevated in OSA patients, especially those with hypertension [99].

Yamazaki et al. has shown that serum MMP-2 was significantly associated with level of plasma brain natriuretic peptide (BNP) in patients with heart failure [100]. BNP is a cardiac hormone, which is a predictor of risk of death in patients with heart failure or cardiac dysfunction [101].

It has been reported that plasma MMP-2 levels increased after myocardial infarctions [102] and after coronary artery bypass graft surgery [103]. Thus, MMP-2 may also be associated with coronary artery disease in OSA.

The mechanical stress on heart in OSA (related to negative intrathoracic pressure) and hypoxemia can lead to ventricular hypertrophy, atrial fibrosis and remodelling [104], which predispose to atrial fibrillation. The upregulation of MMP-2 has been observed in atrial tissue of patients with atrial fibrillation [105].

Changes in circulating MMP-2 have also been reported in stroke patients. The study of Lucivero et al. [106] suggested that MMP-2 can be related to better outcome (plasma MMP-2 level was higher in patients with stable or recovering symptoms in comparison to those who underwent neurological worsening, and MMP-2 negatively correlated with stroke severity). Therefore, MMP-2 might be a prognostic marker for stroke outcomes in OSA.

Furthermore, although the difference in prevalence of diabetes between different groups was not statistically significant in our study (yet increasing along with OSA severity – see Table 5.3), it is important to emphasize the clinical significance of diabetes on CVD development in OSA. OSA and diabetes often coexist, as they share some risk factors such as obesity. [107]. There is a higher risk of atherosclerosis and CVD mortality in OSA patients with diabetes, compared to those without diabetes [108]. Even though it is difficult to define causality in these relationships,

it is possible that MMP-2 is a mediator. It has been shown in animal and cell studies that MMP-2 may contribute to development of diabetic complications (not only cardiopathy [109], but also retinopathy [110] and nephropathy [111]). Thus, MMP-2 action should be explored in larger studies on OSA patients with diabetes.

## **6.2 MMP-9 in serum**

The majority of previous studies on MMPs in OSA have focused on MMP-9, and in our systematic review [15] five out of eight studies examining the relationship between circulating MMP-9 and OSA in adult patients showed that MMP-9 level was elevated in patients with OSA in comparison to patients without OSA or those with less severe OSA [56,70–73].

Fang et al. in their review and meta-analysis on MMP-9 in OSA [112] confirmed that the peripheral level of MMP-9 was increased in OSA patients, and the increase was relevant to OSA severity. Different inclusion and exclusion criteria resulted in a higher number of papers included by Fang et al. in their review - seven out of 12 papers showed increased MMP-9 levels in adult OSA patients [112]. In contrast, four reported no difference in MMP-9 level between patients with more severe OSA in comparison with controls or those with less severe OSA [74–76,78]; and one article showed that serum MMP-9 level was significantly lower in severe OSA than in controls [113].

However, the positive results were not confirmed in this study. There was no significant difference in MMP-9 activity between OSA patients and controls (Figure 5.1), and the difference in MMP-9 activity between groups with different OSA severity did not suggest linear association with oxidative stress related to OSA (Figure 5.2).

Interestingly, only three [74,75,78] (out of 5) studies showing negative results were included in the meta-analysis of Fang et al. [112]. Among the excluded papers, the study of Volná et al. [76] is worth mentioning. They did not report any significant difference in blood MMP-9 concentration in regards to OSA severity, but the pattern observed for different OSA severity was similar to the one observed in our cohort. Specifically, there was a noticeable drop in MMP-9 levels in the moderate OSA group. Both age and BMI were similar between studies. Unfortunately, more detailed comparison is impossible, due to limited data in the study of Volná et al. [76].

It is worth emphasizing that most of previous studies were methodologically flawed in that they measured MMP concentration rather than activity. MMPs are produced as inactive zymogens,

thus, in respect to MMP biology, enzyme activity is a far more informative measurement. Among previous studies there are only two that measured MMP activity, and they showed conflicting results. Tazaki et al. [72] showed that serum MMP-9 activity was higher in patients with moderate/severe OSA than those with mild OSA or controls, whereas Nizam et al. [113] showed that serum MMP-9 activity was significantly lower in patients with severe OSA compared to controls. Thus, MMP-9 activity cannot be suggested as a marker of severity based on this study.

Our results showed that serum MMP-9 activity was associated with WBC count, independently of AHI/ODI, age, sex, BMI and CVD (Table 5.8). These results are in accordance with study of Snitker et al. carried out on apparently healthy population suggesting that the main source of circulating MMP-9 are WBC [114].

Causation cannot be inferred from the results of any observational study, however, the putative mechanism leading to changes in circulating MMP-9 activity seems to be inflammation (measured as WBC count [115]), rather than oxidative stress itself. MMP-9, unlike MMP-2, is highly inducible by inflammatory processes [34]. The associations between OSA severity and total WBC and neutrophil counts have been previously shown [116]. Along this line of thinking, WBC count (or inflammation it represents) could be on a causal pathway linking OSA severity with MMP-9 level. However, multicollinearity between AHI/ODI and WBC was not found in our analysis (VIF values for AHI and WBC were 1.15 and 1.36, respectively).

Kaditis et al. also showed that plasma MMP-9 was significantly associated with CRP (indicator of inflammation) and BMI, but not with AHI [78]. The positive correlation between CRP and serum MMP-9 level was also shown by Ye et al. [73] (however, MMP-9 was associated with AHI). Except for the studies where patients with systemic or respiratory inflammation were excluded [56,72], the authors did not address the potential confounding of inflammation in their analysis on MMP-9 in OSA patients.

It is likely that inflammation via MMP-9 could be a link between OSA and CVD. Neutrophils – the most numerous type of WBC - are a measure of chronic inflammatory state, and even within normal range, they were shown to be associated linearly with risk of developing myocardial infarction, ischemic stroke, heart failure and peripheral artery disease [117], all of which are seen in OSA patients. Oxidative stress related to OSA can modulate WBC action, leading to release of MMP-9 [71]. MMP-9 in turn can contribute to development and progression of atherosclerosis [118] – a condition underlying many cardiovascular disorders.

### **6.3 MMPs in urine**

Nocturnal hypoxemia has been previously shown to be associated with kidney injury [20] and loss of kidney function [19] in OSA patients. The detrimental effect of OSA on the kidneys may be either due to direct effect of intrarenal hypoxia, or as an indirect result of oxidative stress, inflammation and activation of renin-angiotensin-aldosterone system (RAAS). RAAS facilitate systemic hypertension and glomerular hyperfiltration, which can lead to progression of glomerular damage [119].

There was no evidence of tubular injury (measured as NGAL) in our cohort, which is in accordance with the study carried out by Maski et al. [120]. The eGFR values of our study subjects did not indicate hyperfiltration, however, we have had only one-time measurement of eGFR, which could have been assessed during the intermediate state – before or after a supraphysiological increase in GFR.

It needs to be kept in mind that eGFR can be within normal range even in the presence of considerable loss of nephron mass [121]. In order to assess the level of glomerular damage, ACR was calculated. In our cohort, in more severe OSA, a higher ACR was observed; which is in accordance with the results showing that OSA is associated with increased urinary albumin excretion [122]. In this study, urinary MMP-2-to-creatinine was significantly associated with ACR, even after adjustment for eGFR, serum MMP-2, age, sex, BMI, and presence of hypertension and diabetes (Table 5.14).

Given that urinalysis is an inexpensive, safe and common test, urinary measurements could be an easily accessible source of information useful for OSA management. To the best of our knowledge, this is the first study investigating MMPs in urine samples of OSA patients. The results have shed some light on urinary MMPs activity, showing that urinary MMP-2 level in OSA was associated with glomerular injury (measured as ACR), independently of hypertension and diabetes.

As causality cannot be established directly in cross-sectional studies, it is uncertain if urinary MMP-2 activity is elevated as a result of glomerular damage, or if it is MMP-2 that causes the damage. The latter idea is consistent with some previous studies showing that MMP action can modulate renal microvascular permeability (through cleavage of type IV collagen - the main component of glomerular basement membrane), and mediate glomerulosclerosis and tubulointerstitial fibrosis [123].

We found a trend ( $p = 0.084$ ) towards higher urinary MMP-9 activity in OSA patients compared to controls (Figure 5.1), and the difference was more prominent and significant between severe OSA and controls (Figure 5.2). However, regression analysis did not confirm any significant associations with neither AHI nor any measurements of kidney function (eGFR, ACR, NGAL) (data not shown).

Alteration in urinary MMP levels has been previously shown in acute and chronic renal injury [123–125], and the results of our study provide a link between urinary MMP-2 and nephropathy (namely glomerular damage) in OSA.

OSA often occurs in the presence of other comorbidities, and both OSA and its comorbid conditions often share common risk factors (such as age or obesity) and may have overlapping pathophysiology (oxidative stress and inflammation among others) [107]. It has been suggested that OSA and chronic kidney disease are potentially related in a bidirectional fashion [126]. Thus, similarly to analysis in serum, it is very difficult to discern the individual factor contributing to changes in urinary MMP levels in OSA.

#### **6.4 Study limitations**

Although this study has provided important insight into association between MMP activity and OSA, there are several limitations that should be acknowledged. First, the effect size was lower than expected and, in several cases, the achieved power was not sufficient. Second, patient self-reporting of cardiovascular comorbidities may be listed as the possible limitation, however, it was verified by chart review, which includes all the medical history and work-up from referring physicians.

Further, the selection of various control groups should be discussed. The study subjects were chosen from those referred to the Sleep Disorders Center and may not be representative of general population. The choice of different control groups could lead to different associations between MMPs and OSA. In our case, the associations may be an underestimate. The reason is that because the patients were referred to the clinic, they may be experiencing another sleep condition and would be more likely to have an elevated MMP due to that underlying condition or any other source of inflammation/oxidative stress than those in the general population, or the reason for being referred to the clinic may be related to outcome (Berkson bias). An alternative control population could have been healthy controls from general population. This would allow

for a comparison of MMP in OSA patients to a group with no underlying conditions and allow a true comparison of background MMP. However, this control group would probably differ from those who were referred to the clinic. Thus, their selection would be challenging in order to not introduce any additional bias. The differences in age, body mass/BMI and prevalence of CVD seen between our cases and controls represent the situation commonly observed in the clinical setting which makes the results more applicable. Given all discussed, in future studies it would be useful to consider multiple control groups (“healthy” control in addition to ours).

Also, even though we controlled for many plausible confounders, the results of any observational studies need to be interpreted keeping in mind the possibility of residual confounding.

## **6.5 Strengths of the study**

This study differs from others on this subject in several aspects: it has a larger sample size (N = 150 including 124 OSA patients), performance of in-lab PSG in each subject, classification of OSA according to American Academy of Sleep Medicine criteria, and controlling for potential confounders.

The previous studies did not control for WBC count, which could have led to unreliable results as leukocytes are the main source of circulatory MMP-9 [34,114]. Also, MMP-9 is highly inducible by inflammatory processes, which can mediate increases in WBC counts and MMP-9 release. However, the majority of other studies neither controlled for WBC count, nor for inflammation; except from few in which patients with systemic or respiratory inflammation were excluded [56,72] or those where CRP was an additional independent variable in the analysis [73,78].

Moreover, we measured MMPs activity, unlike other studies measuring MMPs concentration. MMPs are produced as inactive zymogens, thus, in respect to MMP biology, enzyme activity is a far more informative measurement.

## 7 CONCLUSIONS

Consistent with our hypothesis, serum MMP-2 activity was associated with OSA severity/level of hypoxemia in OSA. However, such association did not exist for MMP-9. Serum MMP-9 was associated with WBC count (which reflects inflammation).

There were no differences in MMP activities between OSA patients with CVD and without CVD. However, in stratified analysis, the association between serum MMP-2 and AHI remained significant only for patients with CVD. It may imply a potential link between OSA, MMP-2 and CVD. It suggests MMP-2 should be investigated thoroughly in this subgroup as a potential marker of CVD, although this should also be interpreted with caution, as interaction could not be fully assessed.

Urinary MMPs were not shown to be associated with OSA severity. Urinary MMP-2 in OSA was associated with ACR, independently of hypertension and diabetes. It suggests it could be an indicator of glomerular damage in OSA. However, ACR is a commonly used, approved and recommended measurement in this regard.

An ideal biomarker of OSA should be disease-specific and disease-sensitive (for screening purposes), treatment-responsive (for treatment monitoring), and involved in a causal pathway (for prediction OSA comorbidities) [8]. The plausible biological mechanisms (oxidative stress and inflammation) linking MMPs with OSA and its comorbidities justify the interest and hope given to MMPs by researchers in sleep medicine.

Due to the multifactorial pathophysiology of OSA and its comorbidities, it seems prudent to consider the combination of biomarkers in future research. The associations for serum MMPs found in our study are promising. MMP-2 (associated with AHI/ODI) and MMP-9 (associated with WBC counts) could address different causes underlying OSA, namely oxidative stress and inflammation.

Future studies should be prospective in their design, and include a larger sample size in order to increase statistical power. In addition, subgroup analysis of patients with different CVDs should be carried out. Moreover, the effect of CPAP treatment on MMP activities is yet to be determined.



## 8 REFERENCES

- [1] Ayas NT, Hirsch AAJ, Laher I, Bradley TD, Malhotra A, Polotsky VY, et al. New frontiers in obstructive sleep apnoea. *Clin Sci* 2014;127:209–16. <https://doi.org/10.1042/CS20140070>.
- [2] Benjafield AV, Ayas NT, Eastwood PR, Heinzer R, Ip MSM, Morrell MJ, et al. Estimation of the global prevalence and burden of obstructive sleep apnoea: a literature-based analysis. *Lancet Respir Med* 2019;7:687–98. [https://doi.org/10.1016/S2213-2600\(19\)30198-5](https://doi.org/10.1016/S2213-2600(19)30198-5).
- [3] Peppard PE, Young T, Barnet JH, Palta M, Hagen EW, Hla KM. Increased prevalence of sleep-disordered breathing in adults. *Am J Epidemiol* 2013;177:1006–14. <https://doi.org/10.1093/aje/kws342>.
- [4] Government of Canada SC. Sleep Apnea in Canada, 2016 and 2017 2018. <https://www150.statcan.gc.ca/n1/pub/82-625-x/2018001/article/54979-eng.htm> (accessed October 21, 2019).
- [5] Canada PHA of. What is the Impact of Sleep Apnea on Canadians? *Gcnws* 2010. <https://www.canada.ca/en/public-health/services/chronic-diseases/sleep-apnea/what-impact-sleep-apnea-on-canadians.html> (accessed October 21, 2019).
- [6] Skomro RP, Kryger MH. Clinical presentations of obstructive sleep apnea syndrome. *Prog Cardiovasc Dis* 1999;41:331–40. <https://doi.org/10.1053/pcad.1999.0410331>.
- [7] Wang X, Ouyang Y, Wang Z, Zhao G, Liu L, Bi Y. Obstructive sleep apnea and risk of cardiovascular disease and all-cause mortality: A meta-analysis of prospective cohort studies. *International Journal of Cardiology* 2013;169:207–14. <https://doi.org/10.1016/j.ijcard.2013.08.088>.
- [8] Montesi SB, Bajwa EK, Malhotra A. Biomarkers of Sleep Apnea. *Chest* 2012;142:239–45. <https://doi.org/10.1378/chest.11-2322>.
- [9] Ayas NT, Taylor CM, Laher I. Cardiovascular consequences of obstructive sleep apnea. *Curr Opin Cardiol* 2016;31:599–605. <https://doi.org/10.1097/HCO.0000000000000329>.
- [10] Badran M, Ayas N, Laher I. Cardiovascular complications of sleep apnea: role of oxidative stress. *Oxid Med Cell Longev* 2014;2014:985258. <https://doi.org/10.1155/2014/985258>.
- [11] Lavie L. Oxidative stress in obstructive sleep apnea and intermittent hypoxia – Revisited – The bad ugly and good: Implications to the heart and brain. *Sleep Medicine Reviews* 2015;20:27–45. <https://doi.org/10.1016/j.smr.2014.07.003>.

- [12] Wang W, Sawicki G, Schulz R. Peroxynitrite-induced myocardial injury is mediated through matrix metalloproteinase-2. *Cardiovasc Res* 2002;53:165–74.
- [13] Sawicki G, Leon H, Sawicka J, Sariahmetoglu M, Schulze CJ, Scott PG, et al. Degradation of Myosin Light Chain in Isolated Rat Hearts Subjected to Ischemia-Reperfusion Injury: A New Intracellular Target for Matrix Metalloproteinase-2. *Circulation* 2005;112:544–52. <https://doi.org/10.1161/CIRCULATIONAHA.104.531616>.
- [14] Doroszko A, Polewicz D, Sawicka J, Richardson JS, Cheung P-Y, Sawicki G. Cardiac dysfunction in an animal model of neonatal asphyxia is associated with increased degradation of MLC1 by MMP-2. *Basic Res Cardiol* 2009;104:669–79. <https://doi.org/10.1007/s00395-009-0035-1>.
- [15] Franczak A, Bil-Lula I, Sawicki G, Fenton M, Ayas N, Skomro R. Matrix metalloproteinases as possible biomarkers of obstructive sleep apnea severity - A systematic review. *Sleep Med Rev* 2019;46:9–16. <https://doi.org/10.1016/j.smr.2019.03.010>.
- [16] Sawicki G. Intracellular Regulation of Matrix Metalloproteinase-2 Activity: New Strategies in Treatment and Protection of Heart Subjected to Oxidative Stress. *Scientifica* 2013. <https://doi.org/10.1155/2013/130451>.
- [17] Wei Q, Bian Y, Yu F, Zhang Q, Zhang G, Li Y, et al. Chronic intermittent hypoxia induces cardiac inflammation and dysfunction in a rat obstructive sleep apnea model. *J Biomed Res* 2016;30:490–5. <https://doi.org/10.7555/JBR.30.20160110>.
- [18] Ali MAM, Schulz R. Activation of MMP-2 as a key event in oxidative stress injury to the heart. *Front Biosci (Landmark Ed)* 2009;14:699–716.
- [19] Ahmed SB, Ronksley PE, Hemmelgarn BR, Tsai WH, Manns BJ, Tonelli M, et al. Nocturnal Hypoxia and Loss of Kidney Function. *PLoS One* 2011;6. <https://doi.org/10.1371/journal.pone.0019029>.
- [20] Bulcun E, Ekici M, Ekici A, Cimen DA, Kisa U. Microalbuminuria in obstructive sleep apnea syndrome. *Sleep Breath* 2015;19:1191–7. <https://doi.org/10.1007/s11325-015-1136-8>.
- [21] Young T, Skatrud J, Peppard PE. Risk factors for obstructive sleep apnea in adults. *JAMA* 2004;291:2013–6. <https://doi.org/10.1001/jama.291.16.2013>.
- [22] Bassetti CL, Dogas Z, Peigneux P, editors. *Sleep Medicine Textbook*. 2016th ed. European Sleep Research Society; n.d.

- [23] Casale M, Pappacena M, Rinaldi V, Bressi F, Baptista P, Salvinelli F. Obstructive Sleep Apnea Syndrome: From Phenotype to Genetic Basis. *Curr Genomics* 2009;10:119–26. <https://doi.org/10.2174/138920209787846998>.
- [24] Peres BU, Hirsch Allen AJ, Fox N, Laher I, Hanly P, Skomro R, et al. Circulating biomarkers to identify cardiometabolic complications in patients with Obstructive Sleep Apnea: A systematic review. *Sleep Med Rev* 2019;44:48–57. <https://doi.org/10.1016/j.smr.2018.12.004>.
- [25] Adeseun GA, Rosas SE. The Impact of Obstructive Sleep Apnea on Chronic Kidney Disease. *Curr Hypertens Rep* 2010;12:378–83. <https://doi.org/10.1007/s11906-010-0135-1>.
- [26] Kapur VK, Auckley DH, Chowdhuri S, Kuhlmann DC, Mehra R, Ramar K, et al. Clinical Practice Guideline for Diagnostic Testing for Adult Obstructive Sleep Apnea: An American Academy of Sleep Medicine Clinical Practice Guideline. *JCSM* 2017;13:479–504. <https://doi.org/10.5664/jcsm.6506>.
- [27] Avlonitou E, Kapsimalis F, Varouchakis G, Vardavas CI, Behrakis P. Adherence to CPAP therapy improves quality of life and reduces symptoms among obstructive sleep apnea syndrome patients. *Sleep Breath* 2012;16:563–9. <https://doi.org/10.1007/s11325-011-0543-8>.
- [28] Marin JM, Carrizo SJ, Vicente E, Agusti AGN. Long-term cardiovascular outcomes in men with obstructive sleep apnoea-hypopnoea with or without treatment with continuous positive airway pressure: an observational study. *Lancet* 2005;365:1046–53. [https://doi.org/10.1016/S0140-6736\(05\)71141-7](https://doi.org/10.1016/S0140-6736(05)71141-7).
- [29] Peng W, Yan J, Wan Y, Wang B, Tao J, Yang G, et al. Matrix metalloproteinases: a review of their structure and role in systemic sclerosis. *J Clin Immunol* 2012;32:1409–14. <https://doi.org/10.1007/s10875-012-9735-7>.
- [30] Nagase H, Visse R, Murphy G. Structure and function of matrix metalloproteinases and TIMPs. *Cardiovasc Res* 2006;69:562–73. <https://doi.org/10.1016/j.cardiores.2005.12.002>.
- [31] Jones CB, Sane DC, Herrington DM. Matrix metalloproteinases: a review of their structure and role in acute coronary syndrome. *Cardiovasc Res* 2003;59:812–23. [https://doi.org/10.1016/s0008-6363\(03\)00516-9](https://doi.org/10.1016/s0008-6363(03)00516-9).
- [32] Visse R, Nagase H. Matrix Metalloproteinases and Tissue Inhibitors of Metalloproteinases: Structure, Function, and Biochemistry. *Circulation Research* 2003;92:827–39. <https://doi.org/10.1161/01.RES.0000070112.80711.3D>.

- [33] Birkedal-Hansen H, Moore WG, Bodden MK, Windsor LJ, Birkedal-Hansen B, DeCarlo A, et al. Matrix metalloproteinases: a review. *Crit Rev Oral Biol Med* 1993;4:197–250. <https://doi.org/10.1177/10454411930040020401>.
- [34] Parks WC, Mecham RP, editors. *Matrix Metalloproteinases*. 1998th ed. Academic Press; n.d.
- [35] Sawicki G, Salas E, Murat J, Miszta-Lane H, Radomski MW. Release of gelatinase A during platelet activation mediates aggregation. *Nature* 1997;386:616–9. <https://doi.org/10.1038/386616a0>.
- [36] Fernandez-Patron C, Radomski MW, Davidge ST. Vascular matrix metalloproteinase-2 cleaves big endothelin-1 yielding a novel vasoconstrictor. *Circ Res* 1999;85:906–11. <https://doi.org/10.1161/01.res.85.10.906>.
- [37] McQuibban GA, Gong JH, Tam EM, McCulloch CA, Clark-Lewis I, Overall CM. Inflammation dampened by gelatinase A cleavage of monocyte chemoattractant protein-3. *Science* 2000;289:1202–6. <https://doi.org/10.1126/science.289.5482.1202>.
- [38] Wang W, Schulze CJ, Suarez-Pinzon WL, Dyck JRB, Sawicki G, Schulz R. Intracellular action of matrix metalloproteinase-2 accounts for acute myocardial ischemia and reperfusion injury. *Circulation* 2002;106:1543–9.
- [39] Ali MAM, Cho WJ, Hudson B, Kassiri Z, Granzier H, Schulz R. Titin is a target of matrix metalloproteinase-2: implications in myocardial ischemia/reperfusion injury. *Circulation* 2010;122:2039–47. <https://doi.org/10.1161/CIRCULATIONAHA.109.930222>.
- [40] Cadete VJJ, Sawicka J, Jaswal JS, Lopaschuk GD, Schulz R, Szczesna-Cordary D, et al. Ischemia/reperfusion-induced myosin light chain 1 phosphorylation increases its degradation by matrix metalloproteinase 2. *FEBS J* 2012;279:2444–54. <https://doi.org/10.1111/j.1742-4658.2012.08622.x>.
- [41] Jabłońska-Trypuć A, Matejczyk M, Rosochacki S. Matrix metalloproteinases (MMPs), the main extracellular matrix (ECM) enzymes in collagen degradation, as a target for anticancer drugs. *J Enzyme Inhib Med Chem* 2016;31:177–83. <https://doi.org/10.3109/14756366.2016.1161620>.
- [42] Liu P, Sun M, Sader S. Matrix metalloproteinases in cardiovascular disease. *Canadian Journal of Cardiology* 2006;22:25B–30B. [https://doi.org/10.1016/S0828-282X\(06\)70983-7](https://doi.org/10.1016/S0828-282X(06)70983-7).
- [43] Chang C, Werb Z. The many faces of metalloproteases: cell growth, invasion, angiogenesis and metastasis. *Trends Cell Biol* 2001;11:S37–43.

- [44] Cheung PY, Sawicki G, Wozniak M, Wang W, Radomski MW, Schulz R. Matrix metalloproteinase-2 contributes to ischemia-reperfusion injury in the heart. *Circulation* 2000;101:1833–9.
- [45] Krzywonos-Zawadzka A, Franczak A, Sawicki G, Woźniak M, Bil-Lula I. Multidrug prevention or therapy of ischemia-reperfusion injury of the heart-Mini-review. *Environ Toxicol Pharmacol* 2017;55:55–9. <https://doi.org/10.1016/j.etap.2017.08.004>.
- [46] Borth W. Alpha 2-macroglobulin, a multifunctional binding protein with targeting characteristics. *FASEB J* 1992;6:3345–53. <https://doi.org/10.1096/fasebj.6.15.1281457>.
- [47] Brew K, Nagase H. The tissue inhibitors of metalloproteinases (TIMPs): An ancient family with structural and functional diversity. *Biochim Biophys Acta* 2010;1803:55–71. <https://doi.org/10.1016/j.bbamcr.2010.01.003>.
- [48] Phillipson EA. Sleep Apnea – A Major Public Health Problem. *New England Journal of Medicine* 1993;328:1271–3. <https://doi.org/10.1056/NEJM199304293281712>.
- [49] Qaseem A. Management of Obstructive Sleep Apnea in Adults: A Clinical Practice Guideline From the American College of Physicians. *Annals of Internal Medicine* 2013. <https://doi.org/10.7326/0003-4819-159-7-201310010-00704>.
- [50] Evans J, Skomro R, Driver H, Graham B, Mayers I, McRae L, et al. Sleep laboratory test referrals in Canada: Sleep Apnea Rapid Response Survey. *Can Respir J* 2014;21:e4–10.
- [51] Pan W, Kastin AJ. Can sleep apnea cause Alzheimer’s disease? *Neurosci Biobehav Rev* 2014;47:656–69. <https://doi.org/10.1016/j.neubiorev.2014.10.019>.
- [52] Badran M, Yassin BA, Fox N, Laher I, Ayas N. Epidemiology of Sleep Disturbances and Cardiovascular Consequences. *Can J Cardiol* 2015;31:873–9. <https://doi.org/10.1016/j.cjca.2015.03.011>.
- [53] Somers VK, White DP, Amin R, Abraham WT, Costa F, Culebras A, et al. Sleep Apnea and Cardiovascular Disease: An American Heart Association/American College of Cardiology Foundation Scientific Statement From the American Heart Association Council for High Blood Pressure Research Professional Education Committee, Council on Clinical Cardiology, Stroke Council, and Council on Cardiovascular Nursing In Collaboration With the National Heart, Lung, and Blood Institute National Center on Sleep Disorders Research (National Institutes of Health). *Journal of the American College of Cardiology* 2008;52:686–717. <https://doi.org/10.1016/j.jacc.2008.05.002>.

- [54] Sforza E, Roche F. Chronic intermittent hypoxia and obstructive sleep apnea: an experimental and clinical approach. *Hypoxia* (Auckl) 2016;4:99–108. <https://doi.org/10.2147/HP.S103091>.
- [55] Hopps E, Caimi G. Obstructive Sleep Apnea Syndrome: Links Between Pathophysiology and Cardiovascular Complications. *Clin Invest Med* 2015;38:E362-370.
- [56] Wang S, Li S, Wang B, Liu J, Tang Q. Matrix Metalloproteinase-9 Is a Predictive Factor for Systematic Hypertension and Heart Dysfunction in Patients with Obstructive Sleep Apnea Syndrome. *Biomed Res Int* 2018;2018. <https://doi.org/10.1155/2018/1569701>.
- [57] Ben-Yosef Y, Miller A, Shapiro S, Lahat N. Hypoxia of endothelial cells leads to MMP-2-dependent survival and death. *Am J Physiol, Cell Physiol* 2005;289:C1321-1331. <https://doi.org/10.1152/ajpcell.00079.2005>.
- [58] Greenberg H, Ye X, Wilson D, Htoo AK, Hendersen T, Liu SF. Chronic intermittent hypoxia activates nuclear factor-kappaB in cardiovascular tissues in vivo. *Biochem Biophys Res Commun* 2006;343:591–6. <https://doi.org/10.1016/j.bbrc.2006.03.015>.
- [59] Htoo AK, Greenberg H, Tongia S, Chen G, Henderson T, Wilson D, et al. Activation of nuclear factor kappaB in obstructive sleep apnea: a pathway leading to systemic inflammation. *Sleep Breath* 2006;10:43–50. <https://doi.org/10.1007/s11325-005-0046-6>.
- [60] Bond M, Chase AJ, Baker AH, Newby AC. Inhibition of transcription factor NF-kappaB reduces matrix metalloproteinase-1, -3 and -9 production by vascular smooth muscle cells. *Cardiovasc Res* 2001;50:556–65.
- [61] Yuksel M, Kuzu-Okur H, Velioglu-Ogunc A, Pelin Z. Matrix Metalloproteinase-9 Level and Gene Polymorphism in Sleep Disordered Breathing Patients with or without Cardiovascular Disorders. *Balkan Medical Journal* 2013;30:8–12. <https://doi.org/10.5152/balkanmedj.2012.068>.
- [62] Cao C, Wu B, Wu Y, Yu Y, Ma H, Sun S, et al. Functional polymorphisms in the promoter region of MMP-2 and MMP-9 and susceptibility to obstructive sleep apnea. *Sci Rep* 2015;5. <https://doi.org/10.1038/srep08966>.
- [63] Yalcinkaya M, Erbek SS, Babakurban ST, Kupeli E, Bozbas S, Terzi YK, et al. Lack of association of matrix metalloproteinase-9 promoter gene polymorphism in obstructive sleep apnea syndrome. *Journal of Cranio-Maxillo-Facial Surgery* 2015;43:1099–103. <https://doi.org/10.1016/j.jcms.2015.06.014>.

- [64] Maeder MT, Mueller C, Schoch OD, Ammann P, Rickli H. Biomarkers of cardiovascular stress in obstructive sleep apnea. *Clinica Chimica Acta* 2016;460:152–63. <https://doi.org/10.1016/j.cca.2016.06.046>.
- [65] Freitas IG de A, Bruin PFC de, Bittencourt L, Bruin VMS de, Tufik S. What can blood biomarkers tell us about cardiovascular risk in obstructive sleep apnea? *Sleep Breath* 2015;19:755–68. <https://doi.org/10.1007/s11325-015-1143-9>.
- [66] Lira AB, de Sousa Rodrigues CF. Evaluation of oxidative stress markers in obstructive sleep apnea syndrome and additional antioxidant therapy: a review article. *Sleep Breath* 2016;20:1155–60. <https://doi.org/10.1007/s11325-016-1367-3>.
- [67] Li K, Wei P, Qin Y, Wei Y. Is C-reactive protein a marker of obstructive sleep apnea? *Medicine (Baltimore)* 2017;96. <https://doi.org/10.1097/MD.00000000000006850>.
- [68] Svatikova A, Wolk R, Lerman LO, Juncos LA, Greene EL, McConnell JP, et al. Oxidative stress in obstructive sleep apnoea. *Eur Heart J* 2005;26:2435–9. <https://doi.org/10.1093/eurheartj/ehi440>.
- [69] Moher D, Liberati A, Tetzlaff J, Altman DG, PRISMA Group. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *PLoS Med* 2009;6:e1000097. <https://doi.org/10.1371/journal.pmed.1000097>.
- [70] Hopps E, Canino B, Montana M, Calandrino V, Urso C, Lo Presti R, et al. Gelatinases and their tissue inhibitors in a group of subjects with obstructive sleep apnea syndrome. *Clin Hemorheol Microcirc* 2016;62:27–34. <https://doi.org/10.3233/CH-151928>.
- [71] Tamaki S, Yamauchi M, Fukuoka A, Makinodan K, Koyama N, Tomoda K, et al. Production of inflammatory mediators by monocytes in patients with obstructive sleep apnea syndrome. *Intern Med* 2009;48:1255–62.
- [72] Tazaki T, Minoguchi K, Yokoe T, Samson KTR, Minoguchi H, Tanaka A, et al. Increased levels and activity of matrix metalloproteinase-9 in obstructive sleep apnea syndrome. *Am J Respir Crit Care Med* 2004;170:1354–9. <https://doi.org/10.1164/rccm.200402-193OC>.
- [73] Ye J, Liu H, Li Y, Liu X, Zhu J. Increased serum levels of C-reactive protein and matrix metalloproteinase-9 in obstructive sleep apnea syndrome. *Chinese Medical Journal* 2007;120:1482–6.

- [74] Bonanno A, Riccobono L, Bonsignore MR, Lo Bue A, Salvaggio A, Insalaco G, et al. Relaxin in Obstructive Sleep Apnea: Relationship with Blood Pressure and Inflammatory Mediators. *Respiration* 2016;91:56–62. <https://doi.org/10.1159/000443182>.
- [75] Maeder MT, Strobel W, Christ M, Todd J, Estis J, Wildi K, et al. Comprehensive biomarker profiling in patients with obstructive sleep apnea. *Clin Biochem* 2015;48:340–6. <https://doi.org/10.1016/j.clinbiochem.2014.09.005>.
- [76] Volná J, Kemlink D, Kalousová M, Vávrová J, Majerová V, Mestek O, et al. Biochemical oxidative stress-related markers in patients with obstructive sleep apnea. *Med Sci Monit* 2011;17:CR491-497.
- [77] Hopps E, Lo Presti R, Montana M, Canino B, Calandrino V, Caimi G. Analysis of the correlations between oxidative stress, gelatinases and their tissue inhibitors in the human subjects with obstructive sleep apnea syndrome. *J Physiol Pharmacol* 2015;66:803–10.
- [78] Kaditis AG, Alexopoulos EI, Karathanasi A, Ntamagka G, Oikonomidi S, Kiropoulos TS, et al. Adiposity and low-grade systemic inflammation modulate matrix metalloproteinase-9 levels in Greek children with sleep apnea. *Pediatr Pulmonol* 2010;45:693–9. <https://doi.org/10.1002/ppul.21251>.
- [79] Dantas DA da S, Mauad T, Silva LFF, Lorenzi-Filho G, Formigoni GGS, Cahali MB. The extracellular matrix of the lateral pharyngeal wall in obstructive sleep apnea. *Sleep* 2012;35:483–90. <https://doi.org/10.5665/sleep.1730>.
- [80] Hopps E, Caimi G. Matrix metalloproteinases in metabolic syndrome. *European Journal of Internal Medicine* 2012;23:99–104. <https://doi.org/10.1016/j.ejim.2011.09.012>.
- [81] Kusnierova P, Vsiansky F, Pleva L, Plevova P, Safarcik K, Svagera Z. Reference intervals of plasma matrix metalloproteinases 2, 3, and 9 and serum asymmetric dimethylarginine levels. *Scand J Clin Lab Invest* 2015;75:508–13. <https://doi.org/10.3109/00365513.2015.1057760>.
- [82] Kupai K, Szucs G, Cseh S, Hajdu I, Csonka C, Csont T, et al. Matrix metalloproteinase activity assays: Importance of zymography. *J Pharmacol Toxicol Methods* 2010;61:205–9. <https://doi.org/10.1016/j.vascn.2010.02.011>.
- [83] Ricci S, D'Esposito V, Oriente F, Formisano P, Di Carlo A. Substrate-zymography: a still worthwhile method for gelatinases analysis in biological samples. *Clin Chem Lab Med* 2016;54:1281–90. <https://doi.org/10.1515/cclm-2015-0668>.



- [84] Ramirez TA, Jourdan-Le Saux C, Joy A, Zhang J, Dai Q, Mifflin S, et al. Chronic and intermittent hypoxia differentially regulate left ventricular inflammatory and extracellular matrix responses. *Hypertens Res* 2012;35:811–8. <https://doi.org/10.1038/hr.2012.32>.
- [85] Wang W, Zhang K, Li X, Ma Z, Zhang Y, Yuan M, et al. Doxycycline attenuates chronic intermittent hypoxia-induced atrial fibrosis in rats. *Cardiovasc Ther* 2018;36:e12321. <https://doi.org/10.1111/1755-5922.12321>.
- [86] Ramos P, Rubies C, Torres M, Batlle M, Farre R, Brugada J, et al. Atrial fibrosis in a chronic murine model of obstructive sleep apnea: mechanisms and prevention by mesenchymal stem cells. *Respir Res* 2014;15:54. <https://doi.org/10.1186/1465-9921-15-54>.
- [87] Pamerter ME, Powell FL. Time Domains of the Hypoxic Ventilatory Response and Their Molecular Basis. *Compr Physiol* 2016;6:1345–85. <https://doi.org/10.1002/cphy.c150026>.
- [88] Cloward TV, Walker JM, Farney RJ, Anderson JL. Left ventricular hypertrophy is a common echocardiographic abnormality in severe obstructive sleep apnea and reverses with nasal continuous positive airway pressure. *Chest* 2003;124:594–601.
- [89] Schulze CJ, Castro MM, Kandasamy AD, Cena J, Bryden C, Wang SH, et al. Doxycycline reduces cardiac matrix metalloproteinase-2 activity but does not ameliorate myocardial dysfunction during reperfusion in coronary artery bypass patients undergoing cardiopulmonary bypass. *Crit Care Med* 2013;41:2512–20. <https://doi.org/10.1097/CCM.0b013e318292373c>.
- [90] Lin H-B, Cadete VJJ, Sra B, Sawicka J, Chen Z, Bekar LK, et al. Inhibition of MMP-2 expression with siRNA increases baseline cardiomyocyte contractility and protects against simulated ischemic reperfusion injury. *Biomed Res Int* 2014;2014:810371. <https://doi.org/10.1155/2014/810371>.
- [91] Chakraborty C, Sharma AR, Sharma G, Doss CGP, Lee S-S. Therapeutic miRNA and siRNA: Moving from Bench to Clinic as Next Generation Medicine. *Mol Ther Nucleic Acids* 2017;8:132–43. <https://doi.org/10.1016/j.omtn.2017.06.005>.
- [92] Levey AS, Stevens LA, Schmid CH, Zhang YL, Castro AF, Feldman HI, et al. A new equation to estimate glomerular filtration rate. *Ann Intern Med* 2009;150:604–12. <https://doi.org/10.7326/0003-4819-150-9-200905050-00006>.
- [93] Bolignano D, Donato V, Coppolino G, Campo S, Buemi A, Lacquaniti A, et al. Neutrophil Gelatinase–Associated Lipocalin (NGAL) as a Marker of Kidney Damage. *American Journal of Kidney Diseases* 2008;52:595–605. <https://doi.org/10.1053/j.ajkd.2008.01.020>.

- [94] Mehra R, Benjamin EJ, Shahar E, Gottlieb DJ, Nawabit R, Kirchner HL, et al. Association of nocturnal arrhythmias with sleep-disordered breathing: The Sleep Heart Health Study. *Am J Respir Crit Care Med* 2006;173:910–6. <https://doi.org/10.1164/rccm.200509-1442OC>.
- [95] Lavie L. Obstructive sleep apnoea syndrome – an oxidative stress disorder. *Sleep Medicine Reviews* 2003;7:35–51. <https://doi.org/10.1053/smr.2002.0261>.
- [96] Bradley TD, Floras JS. Obstructive sleep apnoea and its cardiovascular consequences. *Lancet* 2009;373:82–93. [https://doi.org/10.1016/S0140-6736\(08\)61622-0](https://doi.org/10.1016/S0140-6736(08)61622-0).
- [97] Ceron CS, Rizzi E, Guimaraes DA, Martins-Oliveira A, Cau SB, Ramos J, et al. Time course involvement of matrix metalloproteinases in the vascular alterations of renovascular hypertension. *Matrix Biol* 2012;31:261–70. <https://doi.org/10.1016/j.matbio.2012.01.009>.
- [98] Wang Mingyi, Kim Soo Hyuk, Monticone Robert E., Lakatta Edward G. Matrix Metalloproteinases Promote Arterial Remodeling in Aging, Hypertension, and Atherosclerosis. *Hypertension* 2015;65:698–703. <https://doi.org/10.1161/HYPERTENSIONAHA.114.03618>.
- [99] Jin Z-N, Wei Y-X. Meta-analysis of effects of obstructive sleep apnea on the renin-angiotensin-aldosterone system. *J Geriatr Cardiol* 2016;13:333–43. <https://doi.org/10.11909/j.issn.1671-5411.2016.03.020>.
- [100] Yamazaki T, Lee J-D, Shimizu H, Uzui H, Ueda T. Circulating matrix metalloproteinase-2 is elevated in patients with congestive heart failure. *Eur J Heart Fail* 2004;6:41–5. <https://doi.org/10.1016/j.ejheart.2003.05.002>.
- [101] Doust J, Lehman R, Glasziou P. The role of BNP testing in heart failure. *Am Fam Physician* 2006;74:1893–8.
- [102] DeLeon-Pennell KY, Meschiari CA, Jung M, Lindsey ML. Matrix Metalloproteinases in Myocardial Infarction and Heart Failure. *Prog Mol Biol Transl Sci* 2017;147:75–100. <https://doi.org/10.1016/bs.pmbts.2017.02.001>.
- [103] Sokal A, Zembala M, Radomski A, Kocher A, Pacholewicz J, Los J, et al. A differential release of matrix metalloproteinases 9 and 2 during coronary artery bypass grafting and off-pump coronary artery bypass surgery. *J Thorac Cardiovasc Surg* 2009;137:1218–24. <https://doi.org/10.1016/j.jtcvs.2008.11.004>.
- [104] Javaheri S, Barbe F, Campos-Rodriguez F, Dempsey JA, Khayat R, Javaheri S, et al. Sleep Apnea: Types, Mechanisms, and Clinical Cardiovascular Consequences. *J Am Coll Cardiol* 2017;69:841–58. <https://doi.org/10.1016/j.jacc.2016.11.069>.

- [105] Xu Jun, Cui Guanggen, Esmailian Fardad, Plunkett Mark, Marelli Daniel, Ardehali Abbas, et al. Atrial Extracellular Matrix Remodeling and the Maintenance of Atrial Fibrillation. *Circulation* 2004;109:363–8. <https://doi.org/10.1161/01.CIR.0000109495.02213.52>.
- [106] Lucivero V, Prontera M, Mezzapesa DM, Petruzzellis M, Sancilio M, Tinelli A, et al. Different roles of matrix metalloproteinases-2 and -9 after human ischaemic stroke. *Neurol Sci* 2007;28:165–70. <https://doi.org/10.1007/s10072-007-0814-0>.
- [107] Boyer S, Kapur V. Obstructive Sleep Apnea: Its Relevance in the Care of Diabetic Patients. *Clinical Diabetes* 2002;20:126–32. <https://doi.org/10.2337/diaclin.20.3.126>.
- [108] Jean-Louis G, Zizi F, Clark LT, Brown CD, McFarlane SI. Obstructive Sleep Apnea and Cardiovascular Disease: Role of the Metabolic Syndrome and Its Components. *J Clin Sleep Med* 2008;4:261–72.
- [109] Meng L, Uzui H, Guo H, Tada H. Role of SGLT1 in high glucose level-induced MMP-2 expression in human cardiac fibroblasts. *Molecular Medicine Reports* 2018;17:6887–92. <https://doi.org/10.3892/mmr.2018.8688>.
- [110] Mohammad G, Kowluru RA. Matrix Metalloproteinase-2 in the Development of Diabetic Retinopathy and Mitochondrial Dysfunction. *Lab Invest* 2010;90:1365–72. <https://doi.org/10.1038/labinvest.2010.89>.
- [111] Thrailkill KM, Bunn RC, Fowlkes JL. Matrix metalloproteinases: their potential role in the pathogenesis of diabetic nephropathy. *Endocrine* 2009;35:1–10. <https://doi.org/10.1007/s12020-008-9114-6>.
- [112] Fang X, Chen J, Wang W, Feng G, Li X, Zhang X, et al. Matrix metalloproteinase 9 (MMP9) level and MMP9 -1562C>T in patients with obstructive sleep apnea: a systematic review and meta-analysis of case-control studies. *Sleep Medicine* 2020;67:110–9. <https://doi.org/10.1016/j.sleep.2019.11.1247>.
- [113] Nizam N, Basoglu OK, Tasbakan MS, Holthofer A, Tervahartiala T, Sorsa T, et al. Do salivary and serum collagenases have a role in an association between obstructive sleep apnea syndrome and periodontal disease? A preliminary case-control study. *Archives of Oral Biology* 2015;60:134–43. <https://doi.org/10.1016/j.archoralbio.2014.09.006>.
- [114] Snitker S, Xie K, Ryan KA, Yu D, Shuldiner AR, Mitchell BD, et al. Correlation of Circulating MMP-9 with White Blood Cell Count in Humans: Effect of Smoking. *PLoS One* 2013;8. <https://doi.org/10.1371/journal.pone.0066277>.

- [115] Pietzner M, Kaul A, Henning A-K, Kastenmüller G, Artati A, Lerch MM, et al. Comprehensive metabolic profiling of chronic low-grade inflammation among generally healthy individuals. *BMC Med* 2017;15. <https://doi.org/10.1186/s12916-017-0974-6>.
- [116] Geovanini GR, Wang R, Weng J, Jenny NS, Shea S, Allison M, et al. Association between Obstructive Sleep Apnea and Cardiovascular Risk Factors: Variation by Age, Sex, and Race. The Multi-Ethnic Study of Atherosclerosis. *Ann Am Thorac Soc* 2018;15:970–7. <https://doi.org/10.1513/AnnalsATS.201802-121OC>.
- [117] Shah AD, Denaxas S, Nicholas O, Hingorani AD, Hemingway H. Neutrophil Counts and Initial Presentation of 12 Cardiovascular Diseases. *J Am Coll Cardiol* 2017;69:1160–9. <https://doi.org/10.1016/j.jacc.2016.12.022>.
- [118] Papazafiropoulou A, Tentolouris N. Matrix metalloproteinases and cardiovascular diseases. *Hippokratia* 2009;13:76–82.
- [119] Hanly PJ, Ahmed SB. Sleep apnea and the kidney: is sleep apnea a risk factor for chronic kidney disease? *Chest* 2014;146:1114–22. <https://doi.org/10.1378/chest.14-0596>.
- [120] Maski MR, Thomas RJ, Karumanchi SA, Parikh SM. Urinary Neutrophil Gelatinase-Associated Lipocalin (NGAL) in Patients with Obstructive Sleep Apnea. *PLOS ONE* 2016;11:e0154503. <https://doi.org/10.1371/journal.pone.0154503>.
- [121] Tonneijck L, Muskiet MHA, Smits MM, van Bommel EJ, Heerspink HJL, van Raalte DH, et al. Glomerular Hyperfiltration in Diabetes: Mechanisms, Clinical Significance, and Treatment. *J Am Soc Nephrol* 2017;28:1023–39. <https://doi.org/10.1681/ASN.2016060666>.
- [122] Faulx MD, Storfer-Isser A, Kirchner HL, Jenny NS, Tracy RP, Redline S. Obstructive Sleep Apnea Is Associated With Increased Urinary Albumin Excretion. *Sleep* 2007;30:923–9.
- [123] Zakiyanov O, Kalousová M, Zima T, Tesař V. Matrix Metalloproteinases in Renal Diseases: A Critical Appraisal. *Kidney Blood Press Res* 2019;44:298–330. <https://doi.org/10.1159/000499876>.
- [124] Han W, Waikar S, Johnson A, Betensky R, Dent C, Devarajan P, et al. Urinary biomarkers in the early diagnosis of acute kidney injury. *Kidney Int* 2008;73:863–9. <https://doi.org/10.1038/sj.ki.5002715>.
- [125] Tashiro K, Koyanagi I, Ohara I, Ito T, Saitoh A, Horikoshi S, et al. Levels of urinary matrix metalloproteinase-9 (MMP-9) and renal injuries in patients with type 2 diabetic nephropathy. *J Clin Lab Anal* 2004;18:206–10. <https://doi.org/10.1002/jcla.20024>.

[126] Abuyassin B, Sharma K, Ayas NT, Laher I. Obstructive Sleep Apnea and Kidney Disease: A Potential Bidirectional Relationship? *J Clin Sleep Med* 2015;11:915–24. <https://doi.org/10.5664/jcsm.4946>.

## Appendix A. Search details

### 1) Embase – Ovid, Embase Classic+Embase (1947 to 2018 December 05)

1. matrix metalloproteinase/ or metalloproteinase/
2. metalloproteinase\*.mp.
3. mmp\*.mp.
4. metalloproteinase\*.mp.
5. 1 or 2 or 3 or 4
6. sleep disordered breathing/
7. (sleep adj2 (apnea or apnoea or breathing)).mp.
8. "hypopnea OR hypopnoea".mp.
9. (disorder\* adj2 breathing).mp.
10. 6 or 7 or 8 or 9
11. 5 and 10

### 2) Medline via Ovid - Ovid MEDLINE(R) and Epub Ahead of Print, In-Process & Other Manuscripts, Daily and Versions(R) (1946 to 2018 December 05)

1. metalloendopeptidases/ or exp gelatinases/ or exp matrix metalloproteinases/
2. metalloproteinase\*.mp.
3. mmp\*.mp.
4. metalloproteinase\*.mp.
5. 1 or 2 or 3 or 4
6. sleep apnea syndromes/ or exp sleep apnea, obstructive/
7. (sleep adj2 (apnea or apnoea or breathing)).mp.
8. "hypopnea OR hypopnoea".mp.
9. (disorder\* adj2 breathing).mp.
10. 6 or 7 or 8 or 9
11. 5 and 10

## Appendix B. Quality assessment

Author, reference number	Selection (★★★★)	Comparability (★★)	Outcome/Exposure (★★★★)
Bonanno et al. (2016) [74]	★★		★★★
Dantas et al. (2012) [79]	★★★★	★	★★★★
Hopps et al. (2016) [70]	★★		★★★
Kaditis et al. (2010) [78]	★★★★	★★	★★★
Maeder et al. (2015) [75]	★ ★		★★★★
Tamaki et al. (2009) [71]	★★	★★	★★★
Tazaki et al. (2004) [72]	★★	★	★★★
Volná et al. (2011) [76]	★		★★★
Wang et al. (2018) [56]		★	
Ye et al. (2007) [73]	★★★★	★★	★★★

For assessing the quality of studies, the modified Newcastle-Ottawa Scale (NOS) was used: Wells GA, Shea B, O'Connell D, Peterson J, Welch V, Losos M, et al. The Newcastle-Ottawa Scale (NOS) for assessing the quality of nonrandomized studies in meta-analyses. Available from: URL: [http://www.ohri.ca/programs/clinical\\_epidemiology/oxford.asp](http://www.ohri.ca/programs/clinical_epidemiology/oxford.asp) (cited Jan 25, 2019).